Development and efficacy testing of inactivated vaccines against Schmallenberg virus infection in cattle and sheep

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„Alles Wissen und alles Vermehren unseres Wissens endet nicht mit einem Schlußpunkt, sondern mit einem Fragezeichen.“
(Hermann Hesse)

1 Introduction

After its emergence in 2011, Schmallenberg virus (SBV) has spread very fast throughout Europe (Wernike, Conraths et al. 2014). Although it is unclear where the virus came from, its relatives from the *Simbu serogroup* of the family *Bunyaviridae*, genus *Orthobunyavirus*, are well known in other parts of the world, like Asia, Australia and Africa (Coetzer and Tustin 2004). The best-studied one among them probably is Akabane virus (AKAV).

SBV, AKAV and other members of the Simbu serogroup like Aino virus (AINOV), Sathuperi virus and Shamonda virus have been shown to infect different domestic ruminant species, namely cattle, sheep and goats (Inaba, Kurogi et al. 1975, Tsuda, Yoshida et al. 2004, Yanase, Fukutomi et al. 2004, Yanase, Maeda et al. 2005, Wernike, Hoffmann et al. 2013). But also wild ruminants and some non-ruminant species are susceptible. For SBV these are deer, chamois, moose, elk, bison, buffalo, muntjak and alpaca as well as dogs (Sailleau, Boogaerts et al. 2013, Wensman, Blomqvist et al. 2013, European Food Safety Authority 2014). Infection with AKAV has equally been detected in wild ruminants. Furthermore, antibodies have been found in horses, camels, buffaloes and pigs (Cybinski, St George et al. 1978, Davies and Jessett 1985, Huang, Huang et al. 2003, Lim, Kweon et al. 2007, Yang, Kim et al. 2008).

The most striking feature of the clinical picture of both, SBV and AKAV infection, is fetal malformation occurring after infection of immunologically naïve females during a certain stage of pregnancy. The consequences may be abortions, birth of non-viable calves, lambs or kids and subsequent economic losses for the owners. In adult males and non-pregnant females on the other hand the course of the infection is usually subclinical.

Due to the fact that these viruses are transmitted by hematophagous insects (De Regge, Deblauwe et al. 2012, Rasmussen, Kristensen et al. 2012, Elbers, Meiswinkel et al. 2013), especially *Culicoides spp*. midges but also mosquitos for AKAV (Hubalek, Rudolf et al. 2014), disease control is associated with characteristic challenges. The best protection is provided by the immune response of the infected host and high antibody titers can be detected after natural infection. They usually persist over months to years. Especially neutralizing antibodies and probably also cellular immune mechanisms contribute to protection after infection with live virus (McClure, McCullagh et al. 1988, Weber and Elliott 2002). In endemic regions this natural immunity is sufficient to prevent major disease outbreaks (Animal Health Australia 2001, OIE World Organisation for Animal Health 2014). However, virus load, activity and distribution of the vector populations may vary due to numerous reasons and thus natural immunity in mammalian hosts is not always acquired before reproductive maturity. To address such cases, inactivated and live vaccines have been successfully developed and applied for AKAV and AINOV (Kurogi, Inaba et al. 1978, Kurogi, Inaba et al. 1979, Kim, Kweon et al. 2011).

The present work describes how an inactivated vaccine against AKAV and AINOV has been tested for its potential to protect cattle from an SBV-infection. Moreover, it reports on the development of inactivated vaccines for SBV and the efficacy testing conducted with the different vaccine candidates.

2 Literature review

2.1 Taxonomic classification of Schmallenberg virus

Schmallenberg virus (SBV) is a member of the family *Bunyaviridae* which has been created by the International Committee on Taxonomy of viruses (ICTV) in 1975 to encompass the members of the former so-called Bunyamawera serogroup. At that time the family included solely the genus *Bunyavirus*. The characteristics of bunyaviruses were defined as follows: spherical, enveloped virions with a single-stranded, segmented RNA genome which replicate in the cytoplasm of their host cell (International Committee on Taxonomy of Viruses 1975).
As more members of the familiy were discovered in the course of the following years the taxonomic differentiation had to be refined. Between 1981 and 1990 further 4 genera (Nairvorus, Phlebovirus, Hantavirus and Tospovirus) were created (International Committee on Taxonomy of Viruses 1981). Additionally, the genus *Bunyavirus* was renamed into *Orthobunyavirus* in 2002 to avoid confusion between the terms for family and genus (Plyusnin and Elliott 2011).
The allocation of the viruses to their respective genera based on structural features like size of the genomic segments, size of the structural proteins and nucleotide sequence on the 3' and 5' end of the RNA segments (Plyusnin and Elliott 2011). Furthermore, there are some unique biological characteristics inherent in the different genera. The genus *Tospovirus* for example is composed of plant pathogenic viruses while members of the genus *Hantavirus* are pathogenic for mammals and are typically transmitted by rodents. As to the remaining three genera, their members are transmitted by different haematophagous arthropods and may cause disease in a variety of mammal species including humans.
The extent of cross reaction of polyclonal antisera raised against one virus with particles of a second virus in different serological tests, i.e. complement fixation (CF), haemagglutination inhibition (HI) and serum neutralization (SN) allows for differentiation of viruses within the different genera (Kinney and Calisher 1981). The extent of antigenic relationship that can be detected varies depending on the test used. The reason for this is that different assays detect antibodies against different proteins. CF tests, for example, are able to detect antibodies targeting non-surface structural proteins of the virus like the nucleoprotein while HI and SN specifically detect antibodies against surface proteins (Kinney and Calisher 1981). The latter are also important for virus attachment and entry into the cell (Kinney and Calisher 1981). For bunyaviruses, the nucleoprotein structure is highly conserved, allowing for detection of remote relationships. Viruses interacting on this level are allocated to one "serogroup". Importantly, this is not a taxonomic entity which is accepted by the ICTV. Surface glycoproteins on the other hand tend to mutate rather frequently as a response to selective pressure exerted by the vector or the host's humoral immune response (Weber and Elliott 2002). Therefore, cross neutralizing activity or cross-hemagglutination indicates close relationship. Beyond that, nucleotide sequence data is increasingly used for classification of bunyaviruses nowadays – just like in other virus families. Thus, virus species are defined by genome sequence, host range, cell and tissue tropism, pathogenicity, cytopathology and antigenic properties (van Regenmortel and Mahy 2004).

The genus *Orthobunyavirus* for example comprises 48 species at present which are distinguished by cross-neutralization and cross-haemagglutination as well as nucleotide sequence of the small genome segment, which encodes the nucleoprotein (International Committee on Taxonomy of Viruses 2012).
SBV was assigned to the Simbu serogroup due to the genome sequence similarities with Sathuperi virus, Douglas virus, and Shamonda virus (Yanase, Kato et al. 2012). Additional cross-neutralization assays (Goller, Höper et al. 2012) confirmed this relationship on a serological basis as well. Concerning the species affiliation it has been proposed that SBV should be allocated to the species Sathuperi virus (Goller, Höper et al. 2012) .

2.2 Molecular structure and replication of Orthobunaviruses

2.2.1 Structural features

As mentioned above, the members of the family *Bunyaviridae* are characterized by spherical and enveloped virions (International Committee on Taxonomy of Viruses 1975). Particle size is between 80 and 120 nm in diameter (Fields, Knipe et al. 2007). The size of SBV-virions has been determined to be about 100 nm (Wernike, Conraths et al. 2014). The virion is made up of four structural proteins. Two viral glycoproteins called Gn and Gc are embedded into the viral envelope. They are connected by disulphide bonds thereby forming a dimer which protruds from the particle's surface at a length of about 5 to 10 nm (Fields, Knipe et al. 2007). Their combined molecular weight averages out at 150 kDa among orthobunyaviruses with about 114 kDa allotted to Gc and 35 kDa to Gn. Furthermore, orthobunyavirus particles comprise about 2000 nucleoprotein particles per virion with an average molecular weight of about 22 kDa each and about 25 copies of the RNA dependent RNA polymerase (RdRp) – also termed L protein because of its size - with an average molecular weight of 260 kDa each (Elliott 1990, Elliott 2008).
The genome is single-stranded and consists of 3 molecules of ribonucleic acid. Each of these genomic segments encodes at least one of the structural proteins. The small – or S – segment (around 900 nucleotides (nt)) codes for the nucleoprotein (also N protein). The medium – or M - segment (around 4500 nt) codes for the glycoproteins Gn and Gc and the large – or L – segment (approx. 6900 nt) codes for the viral RNA dependent RNA polymerase (RdRp) (Elliott 1990).

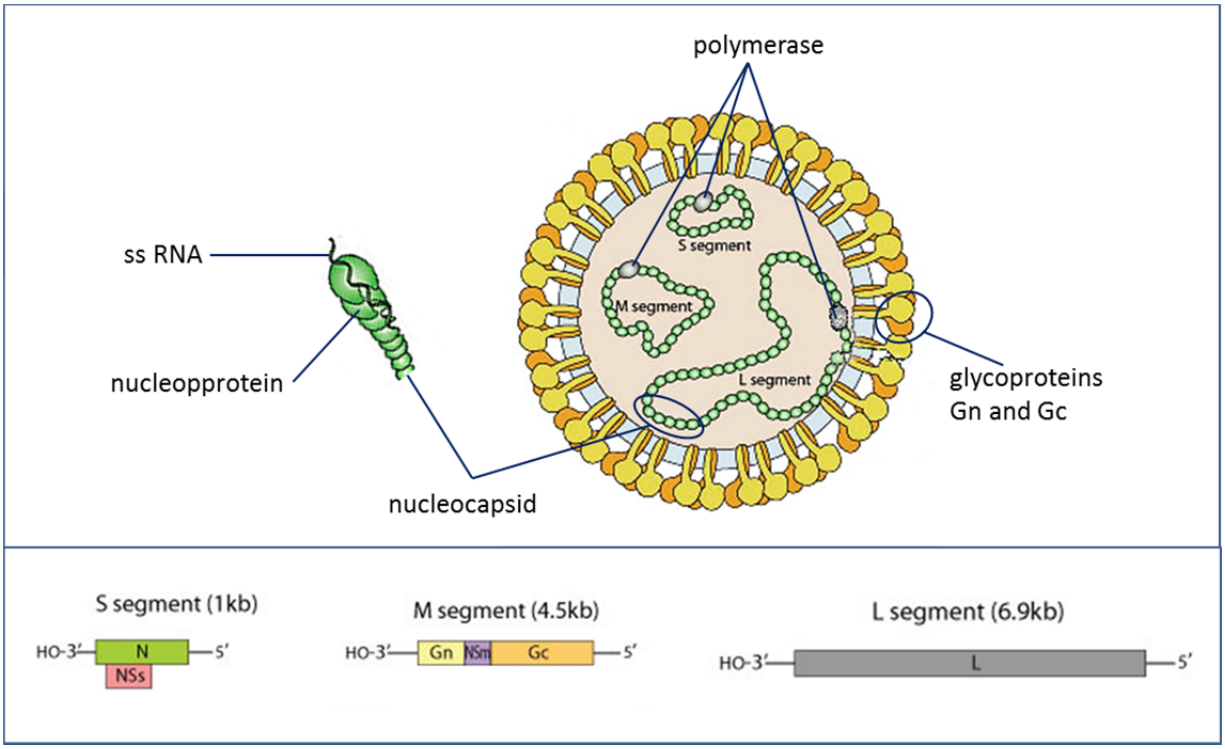

Fig. 1: schematic representation of molecular virion structure of SBV and coding capacitiy of the genomic RNA segments[1].

For SBV, the open reading frames (ORF) of the L, M and S segment comprise 6765 nt, 4212 nt and 702 nt, respectively. The size of the deduced polymerase protein is 2254 amino acids (aa) with a molecular weight of about 261 kDa. The deduced length of the M-derived polyprotein (Gn + NSm + Gc) is 1403 aa with a molecular weight of about 159 kDa (Doceul, Lara et al. 2013). Gn is supposed to comprise aa 1 to 300. The amino acids 301 to 480 are ascribed to NSm, and Gc is represented by aa 481 to 1404 (Fischer, Hoffmann et al. 2013). The nucleoprotein is 233 aa long with a molecular weight of about 26 kDa (Doceul, Lara et al. 2013).

The S and the M segment of most orthobunyaviruses also contain information for two nonstructural proteins, called NSs and NSm, respectively. NSs (~12kDa) is encoded in the form of an overlapping reading frame within the nucleoprotein gene while the sequence for NSm (~16kDa) is located between those of Gn and Gc on the M segment, and the gene product is produced by cleavage of the polyprotein after translation (Elliott 2008, Walter and Barr 2011). NSm seems to be involved in viral replication for some Bunyaviruses (Shi, Kohl et al. 2006). However, it must be assumed that this protein also exerts a number of further functions which have not been elucidated, yet. NSs on the other hand has been proven to antagonize type one interferon synthesis of mammalian host cells thereby promoting viral replication (Eifan, Schnettler et al. 2013). It has been demonstrated that NSs of SBV inhibits mRNA transcription to achieve this (Elliott, Blakqori et al. 2013, Varela, Schnettler et al. 2013, Barry, Varela et al. 2014).

[1] Source: ViralZone www.expasy.org/viralzone, Swiss Institute of Bioinformatics

The coding sequences of each segment are flanked by non-coding regions (NCR) of varying length and sequence. They contain signals which regulate gene transcription, encapsidation and nucleoprotein packaging and they are supposed to influence mRNA translation (Plyusnin and Elliott 2011, Walter and Barr 2011). The terminal 11 nt at both, the 3` and 5` end, are highly conserved and can be used to differentiate between genera (Plyusnin and Elliott 2011). Moreover, the sequence at the 5` end is complementary to the one at the 3` end thereby enabling formation of a so-called panhandle structure. Consequently, the ribonucleoprotein complex is circularized (Plyusnin and Elliott 2011, Walter and Barr 2011). Immediately neighboring the genus specific sequences at either end, there are 3 to 4 nt which are characteristic for the respective serogroup (Plyusnin and Elliott 2011). Furthermore, segment-specific sequences and RNA secondary structures can be found which determine promoter strength.

2.2.2 Replication

The cellular receptor of most bunyaviruses is not definitely known. The viral molecule responsible for attachment to mammalian cells is probably mainly the Gc glycoprotein while Gn is assumed to play a major role for insect cells (Elliott 2008). After endocytosis the ribonucleoprotein is released into the cytoplasm by fusion of the viral and lysosomal membranes. For La Crosse Virus and Bunyamawera virus (BUNV) Gn and Gc have been shown to be involved in this process by ph-dependent conformational changes (Fields, Knipe et al. 2007, Plyusnin and Elliott 2011, Walter and Barr 2011). In the cytoplasm the viral RNA-polymerase then starts to transcribe and replicate the genomic RNA. Several conserved motifs characteristic for polymerase molecules have been identified by comparison with RdRps of other RNA viruses. However, it is unclear if the L-protein of bunyaviruses is the sole component of the functional polymerase complex (Walter and Barr 2011). Experimental results indicate the involvement of a cap snatching mechanism in transcription. This means that 5' caps are cleaved from cytoplasmic host mRNAs and used as primers for the viral transcripts (Elliott 2008, Walter and Barr 2011). Capping of viral mRNA at the same time enables their translation by cellular ribosomes. As described above transcription is probably controlled by specific sequence motifs in the terminal parts of the RNA segments. Transcription termination seems also to be dependent on specific complementary sequences in the 3' and 5' segment ends. One hypothesis for this is that either the template RNA or the nascent RNA product base-pair in this location and thereby inhibits polymerase activity (Plyusnin and Elliott 2011, Walter and Barr 2011). First studies on the structure and function of terminal genomic sequences of SBV, Shamonda virus and Sathuperi virus have proven that they apply cap snatching for their replication. Moreover, insights were gained in the mechanisms of transcription initiation and transcription termination applied by these viruses (Coupeau, Claine et al. 2013).

After some time of transcription, the polymerase changes affinity and the enzyme starts to replicate the viral genome. It is hypothesized that the amount of newly synthesized N protein and an interaction of the viral polymerase with cellular proteins might play a role in this

change. The major difference between transcription and replication is that host mRNA caps are needed to produce transcripts and RNA transcripts do not reach the same length as genomic RNA – they remain about 40 to 100 nt shorter in orthobunyaviruses (Plyusnin and Elliott 2011, Walter and Barr 2011).

Newly synthesized nucleoproteins and viral genomic RNAs accumulate in the cytoplasm where both, protein-protein interactions between nucleoproteins and nucleoprotein-RNA, interactions which depend on sequence- and structure of the molecules, lead to ribonucleoprotein formation (Plyusnin and Elliott 2011). Importantly, association of nucleoproteins with RNA molecules is needed for transcription, RNA synthesis and correct RNP packaging. Correct packaging of genomic segments, which includes differentiation between viral and host RNA and packaging of correct numbers and types of segments, is apparently also influenced by specific sequences in the NCRs (Plyusnin and Elliott 2011). It is furthermore noteworthy that neither genomic viral RNA nor the full-length antigenomic RNA (replicative intermediate RNA) is ever found "naked" (not encapsidated by nucleoproteins) within cells (Plyusnin and Elliott 2011, Walter and Barr 2011). First studies have revealed that the nucleoproteins of SBV are able to form tetramers and hexamers. The different oligomeric forms probably constitute different functional stages during the viral replication cycle when the RNA binding cleft is either exposed to or hidden from interaction with cellular proteins (Dong, Li et al. 2013).

Translation of the M segment mRNA takes place in the endoplsmatic reticulum (ER). The polyprotein is inserted into the membrane and cleaved there cotranslationally while the Gn-protein acts as a chaperone for Gc, ensuring correct folding. The two transmembrane glycoproteins finally form a heterodimer which is stabilized by several intra- and intermolecular disulfide bonds. A specific retention signal within the Gn transmembrane domain is responsible for transport of the dimer to the Golgi. Tubular membrane structures which connect Golgy, ER and mitochondria form so-called virus factories where viral assembly occurs. Interactions between nucleoproteins, polymerase molecules and the cytoplasmic tails of Gn and Gc drive virion morphogenesis (Fields, Knipe et al. 2007, Walter and Barr 2011). For BUNV it has been shown that the nonstructural NSm protein is also involved in this process by integration into the Golgi membrane and interaction with the Gn cytoplasmic tail. Consequently, deletion of specific protein domains of NSm impairs formation of virus like particles (Shi, Kohl et al. 2006). Furthermore, NSm seems to be involved in maturation of the viral glycoproteins of BUNV (Plyusnin and Elliott 2011). As mentioned above, however, it must be assumed that there are more functions to NSm which have not been discovered, yet.

After budding into the Golgi cisternae the virions leave the Golgi via secretory vesicles which are transported to the outer cell membrane and release their contents into the extracellular space.

2.3 Epidemiology

2.3.1 Host spectra and transmission: Akabane virus and Schmallenberg virus

Beside SBV, AKAV is the Simbu virus which has been studied in most detail. It has been isolated from different midge and mosquito species of the genera Culicoides, Culex, Aedes and Anopheles in Japan, Australia and Africa (Jennings and Mellor 1989, Inaba and Matumoto 1990). Its host spectrum among vertebrates is wide. Infection causes considerable economic losses in naive cattle (Kirkland, Barry et al. 1983), sheep (Parsonson, Della-Porta et al. 1977) and goats (Kurogi, Inaba et al. 1977). Antibodies have furthermore been detected in horses (Cybinski, St George et al. 1978, Davies and Jessett 1985, Yang, Kim et al. 2008), pigs (Huang, Huang et al. 2003, Lim, Kweon et al. 2007), camels (Cybinski, St George et al. 1978, Davies and Jessett 1985) and buffalos (Cybinski, St George et al. 1978). Additionally, many wild animal species in Africa have been tested positive for neutralizing anti-AKAV antibodies. These include zebra, different antelope and gazelle species, wildebeest, giraffe, hippo, warthog and elephant (Davies and Jessett 1985, Coetzer and Tustin 2004).

SBV parallels AKAV in many of these aspects. SBV-genome could be detected in bison, deer, moose, buffalo, alpaca (European Food Safety Authority 2013), and elk (Larska, Krzysiak et al. 2013). Fallow deer, roe deer, red deer, llama, chamois, moufflon, reindeer, elk, anatolian water buffalo, sika deer, muntjak and wild boar were positive for SBV-specific antibodies (European Food Safety Authority 2014). Two investigators also reported on antibodies found in dogs (Sailleau, Boogaerts et al. 2013, Wensman, Blomqvist et al. 2013) and in one of these cases SBV-RNA could even be detected in brain samples from a malformed puppy (Sailleau, Boogaerts et al. 2013). Additionally, SBV-specific neutralizing antibodies could be detected transiently in domestic pigs after experimental infection (Poskin, Van Campe et al. 2014). Finally, there is no indication that infection of humans is possible as even highly exposed persons were found seronegative (Ducomble, Wilking et al. 2012, Reusken, van den Wijngaard et al. 2012).
Transmission between mammalian hosts occurs mainly via biting midges. Especially midges of the genus *Culicoides* have been observed to contain SBV-genome (De Regge, Deblauwe et al. 2012, Rasmussen, Kristensen et al. 2012, Elbers, Meiswinkel et al. 2013, Elbers, Meiswinkel et al. 2013, Balenghien, Pagès et al. 2014, Rasmussen, Kirkeby et al. 2014). The precise species that were found SBV-positive were C. obsoletus s.s., C. scoticus, C chiopterus, C. dewulfii, C. pulicaris, and C.nubeculosus. Involvement of other midge species like C. imicola or mosquitoes of the genera Aedes and Culex cannot be ruled out at the moment. Considering current research findings, however, they seem to play only a minor role, if any (Balenghien, Pagès et al. 2014). Moreover, there are no studies to implicate any other arthropods as potential SBV-vectors so far. Finding of SBV-RNA in midge heads without simultaneaous detection of bovine beta actin RNA indicates that especially C. obsoletus s.s. and C. dewulfii are competent vectors for SBV while the role of other species might be limited (Rasmussen, Kirkeby et al. 2014). However, the results obtained after experimental

oral exposure of midges to SBV support the assumption that SBV-replication is also possible at least in C. scoticus and C. nubeculosus midges (Balenghien, Pagès et al. 2014).

Another important mode of transmission is the vertical route, which means that in pregnant females the virus may infect the placental tissue and eventually the fetus. This may disturb the development of fetal organs and potentially leads to malformations or death of the fetus as detailed below (Garigliany, Hoffmann et al. 2012, Herder, Wohlsein et al. 2012, van den Brom, Luttikholt et al. 2012).
Infected bulls may also excrete the virus in their semen over an extended period and this often occurs intermittently (Hoffmann, Schulz et al. 2013, Ponsart, Pozzi et al. 2014, Schulz, Wernike et al. 2014, Van Der Poel, Parlevliet et al. 2014). Thus, virus shedding should be monitored in breeding bulls albeit venereal transmission has so far not been proven.
Finally, oral transmission seems highly improbable based on experimental results although oral, nasal and fecal virus shedding can be detected (Wernike, Eschbaumer et al. 2013).

2.3.2 Geographical distribution of Simbu serogroup viruses

Members of the genus *Orthobunyavirus* can be found world-wide and the same is true for the representatives of the Simbu serogroup (Coetzer and Tustin 2004). Presence of AKAV has been reported especially for Australia (Della-Porta, Murray et al. 1976, Cybinski, St George et al. 1978, Kirkland, Barry et al. 1983) and Japan (Oya, Okuno et al. 1961, Kurogi, Inaba et al. 1975, Konno, Moriwaki et al. 1982), but also for China (Jun, Qingling et al. 2012), Korea (Lee, Park et al. 2002, Lim, Kweon et al. 2007) and Taiwan (Liao, Lu et al. 1996), the Middle East (Taylor and Mellor 1994, Brenner, Tsuda et al. 2004), the Arabian Peninsula (Al-Busaidy, Mellor et al. 1988), Cyprus (Sellers and Herniman 1981), and the African continent (Davies and Jessett 1985, Al-Busaidy, Hamblin et al. 1987). Aino virus which may equally cause the so-called arthrogryposis / hydranencephaly (AG/HE) syndrome (Uchinuno, Noda et al. 1998, Tsuda, Yoshida et al. 2004) has been detected in several Asian countries like Japan and Korea, (Takahashi, Oya et al. 1968, Miura, Inaba et al. 1980, Ishibashi, Shirakawa et al. 1995, Lim, Kweon et al. 2007), South East Asia (Miura, Inaba et al. 1982) and Australia (Cybinski, St George et al. 1978).
Other Simbu serogroup viruses circulating in Asia and Australia are Sathuperi virus (Yanase, Fukutomi et al. 2004), Shamonda virus (Yanase, Maeda et al. 2005), Peaton virus (St George, Standfast et al. 1980, Matsumori, Inai et al. 2002), Douglas virus and Tinaroo virus (Cybinski 1984). Furthermore, Sathuperi virus is present in India (David-West 1972, Yanase, Kato et al. 2012). Shamonda virus, Shuni virus, Simbu virus, Sango virus and Sabo virus have been detected on the African continent (Causey, Kemp et al. 1972, David-West 1972, Yanase, Kato et al. 2012) and the zoonotic Oropouche virus can be found in South America (Pinheiro, Travassos da Rosa et al. 1976).

2.3.3 Emergence and spread of SBV

In summer and autumn 2011, unspecific signs of disease occurred cumulatively in cattle herds in Germany, the Netherlands and Belgium, the cause of which could not be revealed by routine diagnostic methods (ProMed-Mail 2012). In October 2011 blood samples were obtained from a cattle farm in North Rhine-Westphalia. Based on these samples the pathogen could subsequently be identified by metagenomic analysis (Hoffmann, Scheuch et al. 2012). A so far unknown orthobunyavirus from the Simbu serogroup had been discovered. Retrospectively, the earliest detections of SBV-infections were reported for Belgium, the Netherlands and Germany. In Belgium SBV-positive Culicoides midges were found in August 2011 (De Regge, Deblauwe et al. 2012). In the Netherlands positive serological results were found in sheep and SBV-RNA was detected in Culicoides midges in August and September 2011 (Elbers, Meiswinkel et al. 2013, Veldhuis, van Schaik et al. 2013). Furthermore, an epizootic of congenital malformations occurred in Dutch sheep in November 2011, which could be attributed to the new pathogen (van den Brom, Luttikholt et al. 2012). The first observation of viremic cattle in a German herd was reported for September 2011 (Wernike, Silaghi et al. 2014). All animals of this herd seroconverted in the course of a few weeks in September and October. In parallel SBV-RNA was detected in Danish midges collected in October 2011 (Rasmussen, Kristensen et al. 2012) and in France SBV-specific antibodies were detected in blood samples from cattle and red deer from October 2011 onwards (Zanella, Raballand et al. 2013, Laloy, Bréard et al. 2014). Surprisingly, several PCR-positive pools of Culicoides midges were found in Northern Italy as soon as September 2011 (Goffredo, Monaco et al. 2013, Monaco, Goffredo et al. 2013). The first cases of fetal malformation in French domestic ruminants occurred in January 2012 (Doceul, Lara et al. 2013). Equally, the first reports on SBV-disease in a Belgian calf and in British lambs were released in January 2012 (Garigliany, Hoffmann et al. 2012, ProMed-Mail 2012). Next, clinical cases in fetuses were reported from Italy and Luxemburg in February 2012 (ProMed-Mail 2012) and from Spain in March 2012. Interestingly, a small number of SBV positive serum samples were also found in Spanish sheep sampled in July and September 2011 (Astorga, Reguillo et al. 2014). However, confirmation of these latter results by neutralization test was not undertaken. Therefore, it cannot be excluded that these results were false positives.

Serological cross-sectional studies yielded further evidence for extensive circulation of SBV in central Europe in the second half of 2011. By the beginning of 2012 more than three thirds of the cattle population had seroconverted to SBV in the Netherlands and Belgium (Elbers, Loeffen et al. 2012, Méroc, Poskin et al. 2013, Veldhuis, van Schaik et al. 2013). All herds contained at least one positive animal. Similar results were obtained for small ruminants. Estimated seroprevalence rates for sheep were 89% in the Netherlands and 84% in Belgium with almost 100% between-herd prevalence. In the Dutch goat population 51% of the animals had seroconverted by that time compared to 41% in Belgium and 37% in Germany (Helmer, Eibach et al. 2013) corresponding with between-herd prevanlences from 81% to 95%. Respective values among Belgian red and roe deer hunted in autumn 2011 were around 43% (Linden, Desmecht et al. 2012).

As a whole, this information indicates that the new virus was introduced into Europe in summer 2011. The place of emergence was most probably located somewhere near the Dutch-German border.

Spread continued quickly in 2012, bulk milk screening and small-scale seroprevalence studies of English cattle herds at the end of 2012 indicated widespread SBV-circulation during 2012 (Humphries and Burr 2012, Nanjiani, Aitken et al. 2013). In Denmark, a considerable increase in SBV-positive midges was found during that year (Rasmussen, Kirkeby et al. 2014). Switzerland reported on first virus detections for July 2012 (Balmer, Vögtlin et al. 2014) and another study revealed that July or August 2012 also were probable introduction dates for SBV in Austria based on serological investigations (Steinrigl, Schiefer et al. 2014). In Sweden a limited number of seropositive animals was found in June 2012. A few months later, at the end of 2012, a large proportion of susceptible animals throughout the country had been infected. The first confirmed SBV-induced malformations were documented in November 2012. Interestingly, positive serological results were obtained from holdings as far north as 65° north (Chenais, Stahl et al. 2013). Similarly, SBV-positive midges were found in Norway during a collection in September 2012 and bulk milk screening conducted in autumn 2012 revealed a seroprevalence of 17% among the country's cattle population (Wisløff, Nordvik et al. 2014). Parallel to these findings, Ireland (Bradshaw, Mooney et al. 2012), Northern Ireland (ProMed-Mail 2012), Poland (Larska, Polak et al. 2013), Denmark and Finland reported SBV-cases during 2012. Additionally, the virus started to circulate in previously unaffected regions in Germany, France, Great Britain (European Food Safety Authority 2012) and Italy (Balenghien, Pagès et al. 2014). Until May 2013 the virus had finally reached Estonia, Lativa, Hungary, Slovenia, Croatia, the Czech Republic (European Food Safety Authority 2013), Greece (Chaintoutis, Kiossis et al. 2013) and Scotland (Anonymous 2013, Anonymous 2013).
SBV was additionally confirmed by PCR in tissues of aborted calves and lambs in Turkey sampled as early as summer 2012 (Yilmaz, Hoffmann et al. 2014).

All of this impressively illustrates the velocity with which SBV-transmission occurred in European livestock populations. This is equally true for between-herd as well as within-herd transmission (Claine, Coupeau et al. 2013, Shaw, Mellor et al. 2013, Wernike, Silaghi et al. 2014). Beyond this, even minimal vector activity seems to be sufficient for dissemination of the pathogen as acute infections could be detected even in winter months (Wernike, Kohn et al. 2013).

Fig 2: Distribution of SBV as determined by reports of affected countries to the European Food Safety Authority. Status as of September 2013 (Wernike, Conraths et al. 2014).

2.4 Pathology and clinical picture

Just like in respect to epidemiological aspects, AKAV-infection and SBV-infection are quite similar to each other in terms of clinical outcome. Non-pregnant females and male animals infected with AKAV hardly develop any clinical signs (Kurogi, Inaba et al. 1977, Kurogi, Inaba et al. 1977). However, there have been reports on cases of encephalomyelitis in adult cows, which could be associated to an AKAV-infection (Lee, Park et al. 2002). The most noticeable consequences in both, AKAV- and SBV-infection, are epidemic abortion, premature birth or dystocia due to damage and malformations of the fetus after transplacental infection. This has been studied for cattle (Kurogi, Inaba et al. 1977, Konno, Moriwaki et al. 1982, Konno and Nakagawa 1982, Kirkland, Barry et al. 1988, Martinelle, Dal Pozzo et al. 2012), sheep (Parsonson, Della-Porta et al. 1977, Hashiguchi, Murakami et al. 1981, Dominguez, Hendrikx et al. 2012, Saegerman, Martinelle et al. 2013) and goats (Kurogi, Inaba et al. 1977, Helmer, Eibach et al. 2013). The occurrence of fetal malformation in other susceptible species, especially non-domestic ones, has not been studied but must be suspected.

The clinical picture of fetal AKAV- and SBV-infection depends on the point of time in gestation at which the infection occurs. The vulnerable period of fetal development in AKAV-infection seems to be between about day 60 and 250 of gestation for cattle (Kurogi, Inaba et al. 1977, Kirkland, Barry et al. 1988) and between day 30 and 50 for sheep (Hashiguchi, Nanba et al. 1979). So far, the critical phase during which transplacental virus transmission is possible has not been determined exactly for SBV. Preliminary results however seem to confirm that data for AKAV can be applied (European Food Safety Authority 2014, Wernike, Holsteg et al. 2014). After AKAV- or SBV-infection during this phase lesions may develop which are mainly characterized by nonpurulent encephalitis or encephalomyelitis as well as myositis (AKAV) or muscle hypoplasia or dystrophy (SBV) (Konno, Moriwaki et al. 1982, Konno and Nakagawa 1982, Herder, Wohlsein et al. 2012, Hahn, Habierski et al. 2013, Herder, Hansmann et al. 2013, Bayrou, Garigliany et al. 2014, Seehusen, Hahn et al. 2014). More precisely, lesions seen in SBV-infected fetuses consist of cystic cavities in the CNS up to hydranencephaly and reduced size of the ventral horns or micromyelia. In skeletal muscle, decrease in number of myofribils and highly variable fibre diameter is seen as well as a lack of mature muscle fibers (muscle hypoplasia). Parts of muscle tissue may be substituted by fibrous connective and adipose tissue (Herder, Wohlsein et al. 2012, Bayrou, Garigliany et al. 2014, Seehusen, Hahn et al. 2014). Additionally, reduced size of the cerebellum (cerebellar hypoplasia) is regularly described (Herder, Wohlsein et al. 2012, van den Brom, Luttikholt et al. 2012, Helmer, Eibach et al. 2013).

Macroscopic changes consist of arthropryposis, scoliosis, lordosis, kyphosis, torticollis and shortened lower jaw (brachignathia inferior) (Herder, Wohlsein et al. 2012, Bayrou, Garigliany et al. 2014). A relatively small percentage of diseased newborns sufferes from nervous disorders like reduced sucking reflex, ataxic movements or the inability to stand or walk (Garigliany, Hoffmann et al. 2012, Helmer, Eibach et al. 2013). In general, the extent of malformation seen varies widely and congenital defects are observed only in a fraction of the infected fetuses. For AKAV this is a maximum of 30 to 40% (Parsonson, Della-Porta et al. 1977, Kirkland, Barry et al. 1988). In comparison, preliminary studies on the impact of SBV found that malformation occurred in about 5% to 30% of newborn ruminants (Dominguez, Hendrikx et al. 2012, Martinelle, Dal Pozzo et al. 2012, Helmer, Eibach et al. 2013, Saegerman, Martinelle et al. 2013, Luttikholt, Veldhuis et al. 2014). In small ruminants often only one lamb of a multiple birth is affected (van den Brom, Luttikholt et al. 2012, Helmer, Eibach et al. 2013).

Moreover, abortion may occur in different stages of gestation from early embryonic death to premature birth of live, dead or mummified fetuses (Martinelle, Dal Pozzo et al. 2012, van den Brom, Luttikholt et al. 2012, Helmer, Eibach et al. 2013, Veldhuis, Santman-Berends et al. 2014). It is furthermore assumed that the bovine fetus is capable of mounting a specific immune reaction starting from a gestational age of 70 to 130 days of gestation (Schultz, Dunne et al. 1973, Kurogi, Inaba et al. 1977, Brown, Schultz et al. 1979, Kirkland, Barry et al. 1988) while fetal lambs are capable of mounting a humoral immune response starting from about day 70 of gestation (Fahey and Morris 1978). If infection takes place after the fetus has acquired immunocompetence, viral replication may be controlled and fetal development continues mostly undisturbed (Bayrou, Garigliany et al. 2014). The result is the birth of a

healthy newborn at full term of pregnancy. Therefore, serological results should be considered in addition to pathological changes if estimating the rate of transplacental infection. For example, a Belgian study found an SBV-infection rate of 28% in healthy calves based on serological screening of pre-colostral blood samples (Garigliany, Bayrou et al. 2012).

In adult animals infection is mostly inapparent. In the beginning of the epidemic, however, reduced milk yield, fever, inappetence and diarrhea were reported (Hoffmann, Scheuch et al. 2012, Martinelle, Dal Pozzo et al. 2012, Veldhuis, Santman-Berends et al. 2014). Furthermore, abortions and birthing problems (dystocia) were observed. The latter often constituted the only symptoms seen in small ruminants but diarrhea, weight loss and reduced milk yield were observed in single cases (van den Brom, Luttikholt et al. 2012, Helmer, Eibach et al. 2013).

For wild ruminants clinical signs of SBV-infection could so far not be documented (Linden, Desmecht et al. 2012, Barlow, Green et al. 2013, Chiari, Sozzi et al. 2014, Fernandez-Aguilar, Pujols et al. 2014, Laloy, Bréard et al. 2014) although SBV-RNA has been detected in an elk calf in Poland once (Larska, Krzysiak et al. 2013). Unfortunately, detailed investigations through close monitoring of free living deer and other wild ruminants are hardly feasible and adequate sample material is difficult to obtain as dead fawns are quickly eaten by scavengers (Linden, Desmecht et al. 2012). Thus, much might be overlooked. On the other hand clinical disease might indeed be absent as mating periods and vulnerable periods of pregnancy may lie outside the active vector period, depending on the ruminant species in question and the habitat (Chiari, Sozzi et al. 2014).

2.5 Diagnostic tools

2.5.1 Tentative diagnosis

As is apparent from the clinical picture described above, infections with AKAV or SBV may be suspected if fertility problems arise in a herd or flock, especially if this includes a significant increase in abortions and birth of malformed young or newborns with neurological disorders (OIE, terrestrial manual). Increased numbers of adult animals with unspecific clinical signs like fever, diarrhea or reduced feed intake and/or milk yield may contribute to the clinical picture of SBV within a holding (European Food Safety Authority 2013). However, a significant proportion of infections in adult target animals is inapparent. Thus, a definitive diagnosis can only be made if either virus or specific antibodies are detected.

2.5.2 Virus detection

Appropriate samples for virological confirmation of SBV-infection are fetal tissues (Bilk, Schulze et al. 2012, De Regge, van den Berg et al. 2013) or serum, plasma or whole blood

from acutely infected adult animals sampled during the first week after infection (Wernike, Eschbaumer et al. 2012). The same applies for AKAV (OIE World Organisation for Animal Health 2014).
After obtaining the samples, the viruses can be detected either by isolation on cultured cells from hamster (BHK 21; also HmLu-1 for AKAV), African green monkey (Vero) or insects (KC; also C6/36 for AKAV) or by RT-PCR (Hoffmann, Scheuch et al. 2012, Doceul, Lara et al. 2013, Wernike, Hoffmann et al. 2013, OIE World Organisation for Animal Health 2014). The latter is the more sensitive method, especially if the available real-time PCR assays are applied. Beyond that, PCR systems are a suitable tool to screen insect vectors (Veronesi, Henstock et al. 2013, Balenghien, Pagès et al. 2014).
Several drawbacks of virus detection have to be considered: (1) Screening for viral RNA in malformed fetuses only yields positive results in a part of cases. This is especially true for SBV-infected calves and less for lambs; probably because the virus is cleared more effectively during the longer gestation period of cattle (Bouwstra, Kooi et al. 2013, De Regge, van den Berg et al. 2013). (2) Viremia in adult animals is very short and the exact time of infection can not be determined easily in the field. Thus, probabiltiy of virus detection in naturally infected livestock is limited. (3) Attempts to isolate virus on cultured cells may fail due to age and quality of the sample as well as due to the susceptibility of the cells.

2.5.3 Antibody detection

Although serological diagnosis lags behind the actual infection, it is advantageous for the reliable assessment of the epidemiological situation on a large scale. E.g. serological screening of sentinel herds is a key feature of AKAV surveillance in Australia (Animal Health Australia 2001). As deduced from experimental infection studies, seroconversion after SBV- or AKAV-exposure may occur as soon as one week after infection but can also need 2 to 3 weeks to develop (Kurogi, Inaba et al. 1977, Parsonson, Della-Porta et al. 1977, Hechinger, Wernike et al. 2013, Wernike, Eschbaumer et al. 2013, Wernike, Hoffmann et al. 2013). Moreover, titers can be detected for quite a long time. For AKAV persistence of antibodies in naturally infected cattle has been determined to be at least 2 years (Inaba and Matumoto 1990). Under experimental conditions persistence of SBV-specific antibody titers in cattle could be proven for at least 2 months (Wernike, Eschbaumer et al. 2013) and serological studies in the field demonstrated that antibody titers remained high for at least 5 months in infected animals (Wernike, Silaghi et al. 2014). On the population level humoral immunity to SBV was shown to persist for at least one (Méroc, Poskin et al. 2013) or even two years after first infection (Elbers, Stockhofe-Zurwieden et al. 2014).
In newborns or aborted fetuses serological investigation is equally useful for diagnosis of SBV (Kurogi, Inaba et al. 1977, van Maanen, van der Heijden et al. 2012). As mentioned before infection of immunocompetent fetuses often causes less macroscopic changes and especially in calves, RNA detection after birth may be hampered by virus clearance. Therefore, serological testing provides a useful addition to virus detection. A blood sample can be drawn directly from the heart in case the fetus is delivered dead. Alternatively, blood

samples may be obtained from newborns. This should be done prior to colostrum intake because offspring from infected mothers acquires passive immunity through colostral antibodies which persist for about 5-6 months (Tsutsui, Yamamoto et al. 2009, Elbers, Stockhofe-Zurwieden et al. 2014). Such passive immunity has to be differentiated from actual infection of young animals.

For detection of AKAV specific antibodies haemagglutination inhibition (HI), neutralization test (NT) or ELISA are recommended (OIE World Organisation for Animal Health 2014).

For SBV, the NT is considered the "gold standard" although protocols differ between laboratories (van der Poel, Cay et al. 2014). The assay detects antibodies with an affinity for the viral Gc-glycoprotein which are able to prevent attachment to and entry into the cell (Akashi and Inaba 1997, Mansfield, La Rocca et al. 2013). Thus, viral replication and subsequent apoptosis cannot take place. The disadvantages of this test are the facts that it is quite laborious and time consuming and possession of an adequate amount of virus stock is mandatory. Furthermore, it is recommended to use only serum with this test in contrast to EDTA blood or plasma (OIE World Organisation for Animal Health 2014). On the other hand it is highly specific and sensitive and allows differentiation between SBV or Akabane and related Simbu serogroup viruses (Kinney and Calisher 1981, Goller, Höper et al. 2012, Hechinger, Wernike et al. 2013).
As an alternative to the NT, different SBV-specific enzyme linked immunosorbent assays (ELISA) for use with serum or plasma samples have become commercially available (IDvet, IDEXX, LSI). Furthermore, an indirect ELISA for use with bulk milk samples has been developed which facilitates large-scale screening of dairy herds (Humphries and Burr 2012, Chenais, Stahl et al. 2013). Finally, a blocking ELISA has been established in 2013 (ID Screen® Schmallenberg virus Competition Multi-species).
The common feature of all SBV-ELISAs presently available is the detection of antibodies targeting epitopes on the viral nucleoprotein. Thus, their specificity is reduced in comparison to the NT, because nucleoprotein structure is much more conserved between Simbu serogroup viruses than the one of the surface proteins (Saeed, Li et al. 2001, Goller, Höper et al. 2012). The great advantage of the ELISA is reduction of workload enabling laboratories to test large numbers of samples within a relatively short period of time and without the need of handling the actual virus.
A further alternative for serological diagnostics could be the indirect immunefluorescence test (European Food Safety Authority 2013). This was an important tool especially in the early phase of the SBV-epidemic when no automatable ELISA was available (Beer, Conraths et al. 2012). Furthermore, this method allows antigen detection in tissue samples. However, it requires specific tools like specific fluorescence labeled antibodies and fluorescence microscopes and may thus pose more difficulties than a neutralization test while the sensitivity of both tests is similar (Wernike, Eschbaumer et al. 2013).

2.6 Protective measures

2.6.1 Vector control

Basically, there are the following possibilities to control vector populations of biting midges: (1) Reducing the numbers of insects in the environment, or (2) preventing the insects from biting potential host animals. Strategies to achieve these goals are a) destruction of potential insect breeding sites and other habitats, b) application of hormonal (growth regulators) or biological (parasites, pathogens) pesticides or chemical compounds to kill midges in their habitats or in stables and vehicles, c) the use of attractant-equipped insect traps luring the midges away from their actual hosts, d) the application of insecticides to the individual host animal or e) provision of insect proof housing for livestock in order to prevent contact between vector and host. A detailed review on possible techniques for control of Culicoides spp. biting midges has been published with a focus on potential vectors for Bluetongue virus (Carpenter, Mellor et al. 2008).

2.6.2 Management

At least for small ruminants there is the possibility that mating periods may be adjusted in order to avoid overlap of the peak in vector activity and the critical phase of pregnancy. Combined with further protective measures like housing and insecticide treatment this potentially results in significant reduction of clinical cases of disease in a flock (Helmer, Eibach et al. 2013).

2.6.3 Vaccination

A reliable and comparably easy way of prophylaxis is vaccination. Classical approaches for vaccine development comprise the use of whole virus, which is chemically inactivated and thus no longer able to replicate, or the use of replication-competent virus with reduced pathogenicity. Formulations based on the latter are often referred to as “attenuated vaccines” (Kusters and Almond 2008) or “modified live vaccines”(OIE World Organisation for Animal Health 2012). Growing immunological knowledge and the evolution of reverse genetic techniques furthermore fostered the development of a number of new variants. Rift valley fever virus (RVFV, family *Bunyaviridae*, genus *Phlebovirus*) can serve as a good example to illustrate the different approaches to modern vaccine design. As this virus has importance not only as a disease of livestock but also as zoonotic agent, a variety of vaccine candidates have been developed and tested (reviewed in (Ikegami and Makino 2009, Indran and Ikegami 2012)).

2.6.3.1 Inactivated vaccines

For the production of inactivated vaccines the wild-type virus is propagated in cell-culture to obtain sufficient virus content for the future vaccine (Kusters and Almond 2008). Afterwards the virus suspension is mixed with an inactivating agent like formalin or binary ethyleneimine (BEI) (Brown 2001). Formalin inactivates viral particles by cross-linking of proteins. This alters the conformational structure of surface glycoproteins (Brown 2001). Thus, the virus is not able to attach to its cellular receptors and infection of cells is prevented. A big drawback is that this may also affect antigenic structures (Brown 2001). If epitope conformation differs significantly between vaccine virus and wild-type virus, the effectiveness of the host's humoral immune response against wild-type epitopes may be impaired. With BEI this problem is reduced as the inactivating activity of the agent is to a large part associated with structural changes in the viral nucleic acid (Brown 2001). However, protein modifications can not be excluded (Käsermann, Wyss et al. 2001).

2.6.3.2 Modified live vaccines

To obtain a virus strain for production of a modified live vaccine (MLV) several approaches are feasible. Many existing MLV strains were generated by serial passages in cultured cells or laboratory animals until the virus has acquired mutations which render it less virulent for its original host (Kusters and Almond 2008). Mutagenic substances or variations of incubation temperature may be integrated in this procedure (Kurogi, Inaba et al. 1979, Caplen, Peters et al. 1985). Obviously this method depends very much on chance and there is little control over the characteristics the final product will eventually possess. E.g. a sufficient reduction of virulence can not be guaranteed (Hunter, Erasmus et al. 2002). Moreover, as the resulting virus is still capable of replication and host-to-host spread, there is a risk of reversion to the wild-type phenotype (Ikegami and Makino 2009).
In recent decades reverse genetics enabled scientists to create MLV viruses in a much more targeted way. Known virulence factors may be removed from the viral genome. In addition, this allows design of vaccines for the **D**ifferentiation of naturally **I**nfected from **V**accinated **A**nimals (DIVA). In case of bunyaviruses, knock-out of the NSs-gene is known to reduce virulence of the vaccine virus, as the NSs protein is involved in down regulation of the antiviral host response (Eifan, Schnettler et al. 2013, Barry, Varela et al. 2014). It has been already proven that the same is true for SBV (Elliott, Blakqori et al. 2013, Barry, Varela et al. 2014). Although studies which characterize such NSs-deletion mutants in the natural host animal have not been published, yet, they constitute promising candidates for the generation of attenuated SBV-vaccines. Additionally, it has been shown for RVFV that deletion of the NSs sequence can be exploited to differentiate mice vaccinated with NSs -deleted recombinant virus from mice infected with wild-type virus (Lihoradova and Ikegami 2012). The same was shown for sheep (Bird, Albarino et al. 2008). If NSs-deletion mutants are suitable for establishing a DIVA system has to be investigated more closely. Based on results from the experimental characterization of RVFV it seems at least possible. Furthermore, little

is known so far about the function of SBV's second non-structural protein NSm. As more results will become available this could turn out to be another point of attack.
Single viral antigens may also be introduced into another, non-pathogenic virus which then serves as a vector (Kusters and Almond 2008, Indran and Ikegami 2012). Such antigens have to be sufficiently immunogenic. Additionally the vector virus needs to be capable of replication in cell culture or other culture systems to facilitate large-scale production. Some replication in vaccinated individuals furthermore enhances immunogenicity (Papin, Verardi et al. 2011). For RVFV, recombinant vectored vaccines have been constructed from surface glycoprotein genes of RVFV and a vaccinia virus (family *Poxviridae*, genus *Orthopoxvirus*) or a Lumpy skin disease virus (LSDV, genus *Capripoxvirus*) as backbone. Other variants were created and successfully evaluated in animal trials employing Venezuelan equine encephalitis virus and Sindbis virus vectors. Beyond this, Newcastle disease virus (NDV, genus *Avulavirus*, family *Paramyxoviridae*) is considered an appropriate vector which could be used in the future for livestock vaccines in countries where LSDV does not occur (Indran and Ikegami 2012).

2.6.3.3 New ideas: subunit vaccines, virus like particles, replicons, DNA vaccines

For the production of subunit vaccines, selected viral antigens ("subunits" of the virus) may be expressed in cultured cells, bacteria or yeast (Schmaljohn, Parker et al. 1989, Kusters and Almond 2008, de Boer, Kortekaas et al. 2010). After addition of adjuvant to the purified antigen the vaccine is ready for use (Kortekaas, Antonis et al. 2012). Of course the same criteria for selection of appropriate antigens apply as for generation of vectored vaccine virus. Inactivation is dispensable and like in case of RVFV handling of the actual (zoonotic) virus is not necessary (de Boer, Kortekaas et al. 2010). As a variation soluble antigenic components may be produced in the form of virus like particles (VLP) (Ikegami and Makino 2009). Therefore, all structural proteins of the virus which are essential for capsid and membrane formation are expressed in suitable cultured cells while viral nucleic acids are not incorporated. For RVFV, the expression of the nucleoprotein and the two surface glycoproteins Gn and Gc has been shown to result in VLP-formation (Näslund, Lagerqvist et al. 2009), and another study demonstrated that even Gn and Gc alone are able to form VLPs (de Boer, Kortekaas et al. 2010). VLPs are assumed to be immunogenic and offer good stabilization of antigenic structures. Equally to subunit vaccines inactivation procedures and biosecurity measures during production are not necessary (Ludwig and Wagner 2007).
For replicon-based vaccines, DNA plasmids containing complete viral genomic sequences or reading frames of single proteins are transfected into cultured cells where they can be transcribed and translated. In this way infectious viral particles are produced which may lack certain parts of their genome or protein components (Indran and Ikegami 2012). RVFV replicon particles can be generated e.g. by transfecting cultured cells with plasmids containing the S- and L-genomic segments. Additionally, a plasmid containing the open reading frames of Gn and Gc is included. This leads to production of replicon particles containing all viral proteins but only the S and L genomic segments. To accommodate desired vaccine characteristics, modifications of the original viral genome may be performed like deleting the

nonstructural proteins NSs and NSm (Kortekaas, Oreshkova et al. 2011, Dodd, Bird et al. 2012). These particles can infect target cells and replicate to some extent, which is favorable for immunogenicity, but further spread beyond the initially infected host cell is impossible, greatly improving safety in comparison to classical modified live vaccine formulations (Dodd, Bird et al. 2012).

Finally, there are DNA-vaccines which have been developed for the control of human pathogens or zoonotic agents but remain mostly on an experimental level (Donnelly, Wahren et al. 2005, Spik, Shurtleff et al. 2006). Additionally, they may show insufficient immunogenicity (Lagerqvist, Naslund et al. 2009). In this approach genes coding for immunogenic proteins of the targeted pathogen are inserted into a plasmid together with promoter sequences allowing for DNA transcription in mammalian cells and sometimes accompanied by cytokine genes (Operschall, Schuh et al. 1999, Donnelly, Wahren et al. 2005). The plasmid solution may also be injected along with classical adjuvants (Ulmer, DeWitt et al. 1999). In the tissue the DNA plasmid may enter into cells where the encoded information is transcribed and translated. Depending on the route of application muscle cells or even B cells or Langerhans cells may be used as antigen presenting cells, thereby modulating the immune response (Donnelly, Wahren et al. 2005).

2.6.4 Vaccines for orthobunyaviruses

In Australia, Japan and Korea inactivated vaccines as well as modified life vaccines have been developed for prophylaxis of AKAV and AINOV infections. One of these inactivated vaccines for prophylaxis of AKAV and AINOV virus infection has been investigated for its potential as heterologous vaccine against SBV in order to try and provide an effective tool for disease control as soon as possible. The results of this trial are presented in the following chapter. Additionally, development of SBV-specific vaccines was launched quickly after the virus was first discovered in Europe. Part of these efforts is also reported in the present work.

3 Results

The manuscripts are presented in the form accepted for publication and are grouped according to their topic.
Each manuscript has its own reference section formatted in the style of the respective journal; references and abbreviations from the manuscripts are not included in the relevant sections at the end of this document. Figures and tables are numbered individually within each manuscript.

Evaluating the protective efficacy of a trivalent vaccine containing Akabane virus, Aino virus and Chuzan virus against Schmallenberg virus infection

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Abstract

Schmallenberg virus (SBV), an arthropod borne pathogen, spread rapidly throughout the majority of Europe since 2011. It can cause a febrile disease, milk drop, diarrhea, and fetal malformation in ruminants. SBV, a member of the Simbu serogroup within the genus *Orthobunyavirus*, is closely related to *Akabane virus* (AKAV) and *Aino virus* (AINOV) among others. In the present study, 4 Holstein-Friesian calves were immunized twice four weeks apart with a multivalent, inactivated vaccine against AKAV and AINOV. Another 4 calves were kept as unvaccinated controls. All animals were clinically, serologically and virologically examined before and after challenge infection with SBV. AKAV- and AINOV-specific neutralizing antibodies were detected one week before challenge infection, while SBV-specific antibodies were detectable only thereafter. SBV genome was detected in all vaccinated animals and 3 out of 4 controls in serum samples taken after challenge infection. In conclusion, the investigated vaccine was not able to prevent an SBV-infection. Thus, vaccines for other related Simbu serogroup viruses can not substitute SBV-specific vaccines as an instrument for disease control.

Introduction

Schmallenberg virus (SBV), a member of the Simbu serogroup within the genus *Orthobunyavirus*, family *Bunyaviridae*, emerged in Europe in autumn 2011 [1]. Thereafter it spread rapidly throughout large parts of the continent [2]. Blood sucking insects, particularly midges of the *Culicoides obsoletus complex*, are involved in the transmission of the pathogen [3-5]. In adult cattle, sheep and goats mild febrile disease accompanied by reduction of milk yield may be observed, sometimes associated with diarrhea [6]; inapparent infection may occur as well. Fetal infection during a critical phase of pregnancy may lead to damage of the central nervous system and the musculoskeletal structures [7, 8]. Stillbirth or birth of weak calves, lambs or kids, abortion and dystocia are the possible consequences [6, 9]. Experimental infection of cattle resulted in RNAemia for a few days and infection of diverse tissues throughout the body of the host [1, 10].

SBV is closely related to *Sathuperi virus* (SATV) and *Douglas virus* (DOUV) [11, 12], and it was demonstrated that SBV-specific antibodies are able to neutralize infectivity of DOUV, SATV and *Aino virus* (AINOV) in vitro [11]. Additionally, serological cross-reactions between *Akabane virus* (AKAV), AINOV, DOUV, SATV and *Shamonda virus* (SHAV) were described previously [13]. These cross-reactions were detected in complement fixation tests

(CFT), not in neutralization tests. Consequently, the contribution of such antibodies to a protective effect might be limited. Beyond that, AINOV and AKAV cause symptoms in ruminants which are similar to those of an SBV-infection [14, 15], and vaccines have been developed for disease control [16, 17]. *Chuzan virus* (CHUV, family *Reoviridae*, genus *Orbivirus*) is another teratogenic pathogen of ruminants which occurs in Asia [18, 19]; it has been included into a multivalent vaccine together with AKAV and AINOV. In the present study, the possible cross-protection of this multivalent vaccine against a subsequent challenge infection with SBV was investigated.

Materials and methods

Experimental design

The experimental protocol was reviewed by a state ethics commission and has been approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Vorpommern, Rostock, Germany, ref. LALLF M-V TSD/7221.3-1.1-004/12).

Eight SBV-naive female Holstein-Friesian calves were divided in 2 groups of 4 individuals. The average age was 9.4 months at the first vaccination. The animals were housed under BSL 3 conditions during the entire study to prevent a natural SBV-infection.

Animals of group 1 (C01-C04) were immunized intramuscularly twice 4 weeks apart with 3 mL of a trivalent inactivated vaccine for AKAV, AINOV and CHUV (Nisseiken Bovine Abnormal Parturition Trivalent Inactivated Vaccine, Nisseiken Co., Ltd, Japan). The efficacy and safety of this vaccine have been investigated previously [16]. The second group (C05-C08) was used as unvaccinated control. Injection sites were monitored daily for 4 days after both vaccinations.

Six weeks after the first vaccination all animals were inoculated subcutaneously with 2 × 0.5 mL of an SBV field strain that was only passaged in cattle [10]. After the challenge infection the animals were monitored for clinical signs by veterinarians for eight days.

Rectal body temperature was recorded daily. Blood samples were collected weekly, starting from day 7 after the first vaccination (7 days post vaccination, dpv), as well as daily on the 8 days following challenge. Serum samples were analyzed with a commercially available SBV antibody ELISA (ID Screen® Schmallenberg virus Indirect, IDvet, France) and in standard microneutralization tests (SNT) against SBV, AKAV and AINOV [20].

Samples of spleen, tonsils, and mesenteric and mandibular lymph nodes were taken at autopsy and homogenized in 1 mL of Minimum Essential Medium (MEM).

RNA extraction and real-time RT-PCR

RNA was extracted from serum and tissue samples using the MagAttract Virus Mini M48 Kit (Qiagen, Germany) according to the manufacturer's recommendations.

SBV genome load was determined by an SBV-specific reverse transcription real-time PCR (real-time RT-PCR) as described previously [21] with an external standard based on the small (S) genome segment.

Results

Clinical observation and pathology

None of the animals showed any signs of clinical disease. Body temperatures were within a normal range for all animals. The measured temperatures never exceeded 39.5 °C. Additionally, no adverse side effects were observed following either vaccination. Autopsy did not reveal any significant gross lesions.

Serology

All animals were seronegative for SBV, AINOV and AKAV before first vaccination (Figure 1). In one vaccinated animal first AKAV-specific antibodies could be detected one week after the first vaccination and two weeks prior to challenge, respectively. All immunized animals were SNT-positive for AKAV and AINOV one week before challenge infection. First SBV-specific neutralizing antibodies were detected in two of four animals one week after challenge (Figure 1). Two weeks after challenge infection all four vaccinated animals were positive for SBV, both in SNT and ELISA.

The four control animals remained seronegative for AKAV- and AINOV throughout the study.

SBV-specific antibodies were detected starting from the second week after challenge infection, with three animals being positive in the SNT, and one animal scoring positive in the ELISA. A second control animal showed a positive reaction in the ELISA in the third week after challenge infection. One control animal (C05) remained negative for SBV in both serological assays until the end of the study.

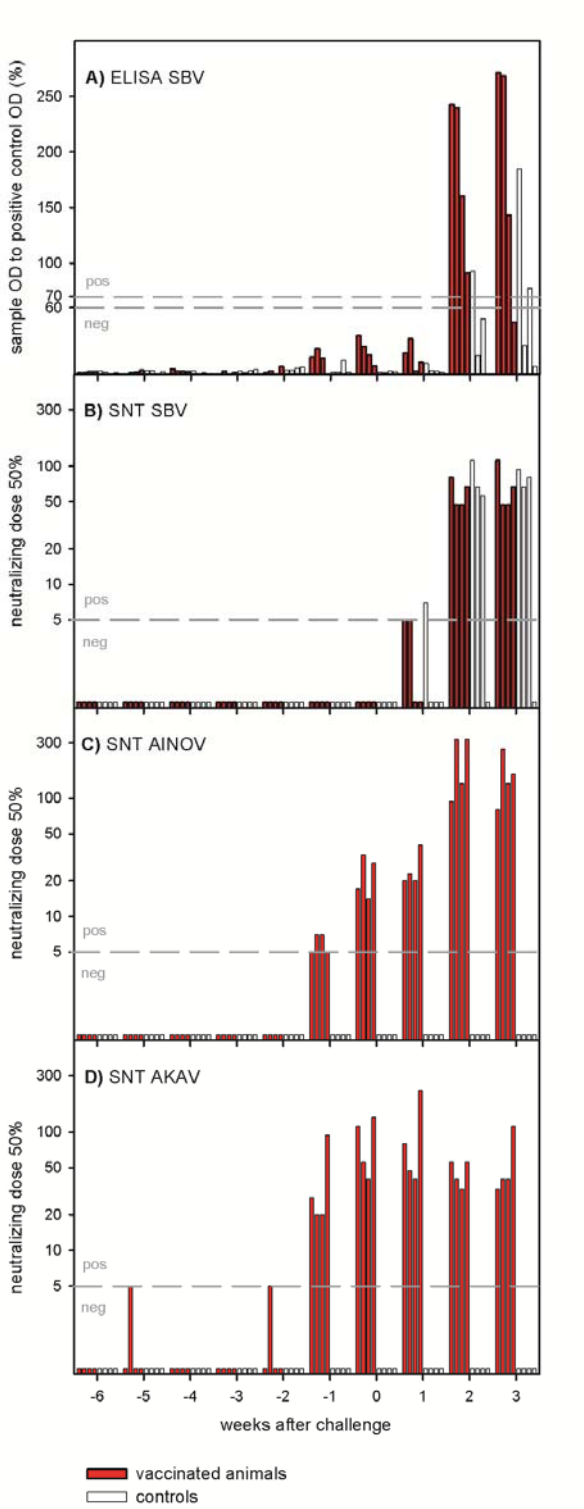

Figure 1 Serology

The animals were vaccinated 6 and 2 weeks before challenge. Bars represent one animal each. Serum samples were tested by a commercially available SBV antibody ELISA (A) and in standard microneutralization tests against SBV (B), AINO (C) and AKAV (D). Horizontal dashed lines indicate the cut-off value of the respective test. The neutralization titers are expressed as reciprocal of the serum dilution showing 50% virus neutralization.

Real-time RT-PCR

All vaccinated animals as well as three of four control animals were positive in the RT-PCR (Figure 2). SBV genome was detected in the serum samples of all vaccinated animals and in all control animals with the exception of C05. In group 1 (vaccinated animals) RNAemia was seen from 2 days post challenge (dpc) infection to 5 dpc in two animals (C03, C04), from 3 dpc to 6 dpc in one animal (C02) and from 3 dpc to 7 dpc in the remaining animal (C01). In group 2 (controls), the animals showed RNAemia from 2 dpc to 5 dpc (C06, C08) and from 1 dpc to 6 dpc (C07), respectively.

Furthermore, SBV genome was detected in all vaccinated animals in the mesenteric lymph nodes (average: 3.89×10^5 genome copies/mg sample material), the spleen samples (average: 1.01×10^5 copies/mg) and mandibular lymph nodes (average: 3.59×10^4 copies/mg). In tonsils of C01 and C03 1.55×10^5 copies/mg and 1.82×10^2 copies/mg were detected.

In control animals C06, C07 and C08, SBV genome was detected in mesenteric lymph nodes (average: 1.66×10^6 copies/mg) and spleens (average: 3.05×10^5 copies/mg). In C07 and C08 mandibular lymph nodes were positive as well (1.36×10^6 copies/mg and 5.08×10^5 copies/mg respectively) and tonsils of C07 contained 4.92×10^2 copies/mg. In samples from calf C05 SBV-genome was not detectable.

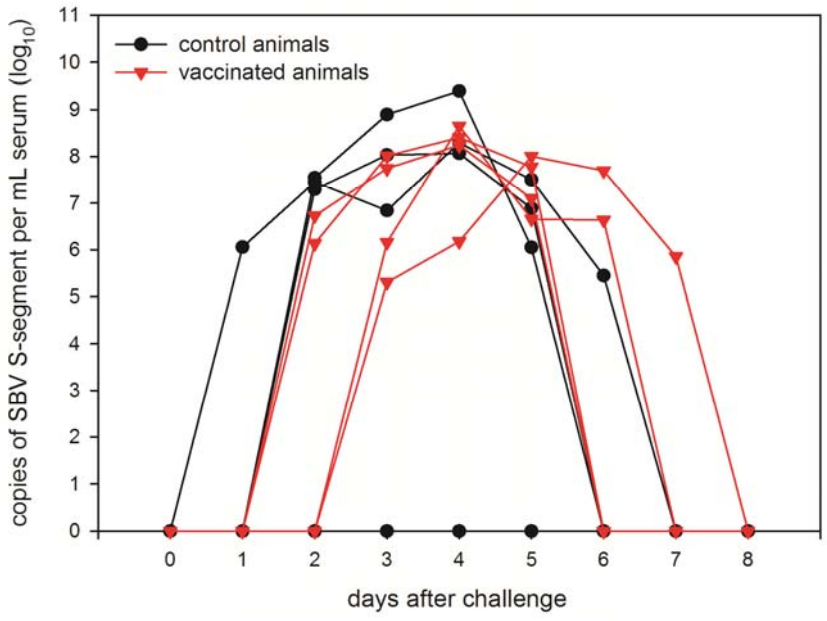

Figure 2 Real-time RT-PCR results for serum samples after challenge infection.

All vaccinated animals (depicted in red) and three out of four control cattle (black) scored positive in the RT-qPCR for several days.

Discussion

Antibodies specific for Simbu serogroup viruses frequently cross-react with more than one other member of this serogroup [11, 13]. Such interaction might also influence viral replication in vivo. After the emergence of SBV in Europe, vaccines against related Simbu viruses, such as AKAV and AINOV, could potentially offer a tool for disease control until an SBV-specific vaccine is ready for use.

Considering the current epidemiological situation vaccination of young female sheep or cattle before their first pregnancy will be an important measure to eliminate the risk of SBV-infection of naïve animals during the vulnerable phase of fetal development. Therefore, calves instead of cows were used in the present study. Data about the effect of vaccination of calves were not published for the vaccine we used but the efficacy of another inactivated AKAV-specific vaccine in calves aged 5 to 10 months has been proven [17]. Additionally, inactivated SBV vaccines are efficacious in calves [22].

The trivalent AKAV/AINOV/CHUV-vaccine applied in the present study has proven its effectiveness [16]. Neutralizing antibodies against AINOV and AKAV could be detected shortly after the second vaccination. For AKAV it has been demonstrated, that a neutralizing titer of 16 in experimental animals prevented RNAemia after infection in comparison to one control animal [16]. Thus, we assume that a mean neutralizing titer of 85 (minimum 40), as detected in the present study, would have provided protection against AKAV infection. For AINOV protective antibody titers could not be determined from the literature. However, the titers detected in the present study are comparable to or even exceeded those given for cattle in earlier studies on this vaccine [16]. Therefore, protection could probably have been expected in case of an AINOV-infection also.

SBV-specific antibodies were detectable only after challenge infection. In the ELISA some low activity was seen for the samples of vaccinated animals in week -1 and 0 (Figure 1). At the same time anti-AKAV and anti-AINOV antibodies started to be detectable in vaccinated animals by neutralization test. Serological cross-reaction between close relatives of SBV (SATV, DOUV, SHAV) and AKAV/AINOV have already been described in CFT [13]. This test also detects non-neutralizing antibodies like anti-nucleoprotein antibodies. As the ELISA that was used for our analysis is based on recombinant SBV nucleoprotein for antibody detection this can explain the results.

Unlike Goller et al. [11] we did not detect cross-neutralization between AINOV and SBV. One possible reason for this discrepancy is the determination of the neutralizing activity of anti-AINOV/AKAV antibodies towards SBV in the present study, but of neutralizing activity

of anti-SBV antibodies towards AINOV and other Simbu viruses by Goller et al. [11]. In contrast to the nucleoprotein-based ELISA, neutralization depends on antibodies binding to viral glycoproteins. This can explain that there was some evidence of cross reactivity in the ELISA but not in the neutralization test. Furthermore, the AINOV/AKAV antibodies in our study were induced by vaccination while the SBV-antiserum used by Goller et al. [11] was collected following SBV-infection.

After inoculation with SBV, viral RNA was present in serum samples of all vaccinated animals for several days and in 7 of 8 animals SBV-RNA was detected in the lymphatic tissues sampled at autopsy. The same was observed in unvaccinated control animals during SBV vaccine studies, whereas vaccination with inactivated prototype SBV vaccines has been associated with reduced RNA load in serum and tissue samples or no detection of SBV genome at all, even if SBV-specific antibody titers were low [22].

Remarkably, highest SBV-genome loads for tissue samples were found in mesenteric lymph nodes in most animals. This is in agreement with results from earlier studies [10]. However, the role of lymphatic tissues in the pathogenesis of SBV infection has not been thoroughly investigated so far and is a topic to which attention should be paid in future research.

It is unknown why one animal (C05) failed to show signs of infection both in serological tests and PCR but similar observations have been made after experimental SBV-infection of sheep [23]. One explanation could be a failed injection, another one a general resistance to SBV-infection of unknown cause.

In conclusion, protection against SBV-infection could not be proven for the multivalent vaccine tested. Thus, vaccines for other related Simbu serogroup viruses can not substitute SBV-specific vaccines as an instrument for disease control.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: KW, MB. Performed the experiments: SH, KW. Analyzed the data: SH, KW. Wrote the paper: SH, KW, MB. All authors read and approved the final manuscript.

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Inactivated Schmallenberg virus prototype vaccines

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Abstract

Schmallenberg virus (SBV), a novel Orthobunyavirus, is an insect-transmitted pathogen and was first described in Europe in 2011. SBV causes a mild transient disease in adult ruminants, but severe foetal malformation and stillbirth were observed after an infection of naive cows and ewes, which is responsible for considerable economic losses. The virus is now widely distributed in Europe, and no vaccines were available to stop transmission and spread.

In the present study, 16 calves and 25 sheep, the major target species of SBV infection, were vaccinated twice 3 weeks apart with one of 5 newly developed, inactivated vaccine candidates. Six calves and 5 sheep were kept as unvaccinated controls. All animals were clinically, serologically and virologically examined before and after challenge infection.

Immunisation with the inactivated preparations resulted in a neutralising antibody response three weeks after the second vaccination without any side effects. The number of animals that seroconverted in each group and the strength of the antibody response were dependent on the cell line used for virus growth and on the viral titre prior to inactivation. Four vaccine prototypes completely prevented RNAemia after challenge infection, a fifth candidate reduced RNAemia considerably. Although further evaluations e.g. regarding duration of immunity will be necessary, the newly developed vaccines are promising candidates for the prevention of SBV-infection and could be a valuable tool in SBV control strategies.

1. Introduction

After the emergence of bluetongue virus serotype 8 (BTV-8) in 2006 [1], another vector-transmitted pathogen affecting domestic and wild ruminants was introduced to Northern Europe in 2011. The novel virus, referred to as “Schmallenberg virus” (SBV), was discovered near the German-Dutch border and thereafter spread rapidly to other European countries [2] and [3]. SBV is a member of the Simbu serogroup within the genus *Orthobunyavirus*; the most related viruses are Sathuperi virus and Douglas Virus [4]. Acute SBV infection causes no or only mild clinical signs including fever, reduced milk production or diarrhoea for a few days. However, an infection of naive cows and ewes during a critical period of pregnancy can lead to severe foetal malformations [5] and [6]; reviewed in [2] and [7].

Insect vectors are responsible for the spread of SBV and viral genome was detected in field-collected *Culicoides spp.* biting midges [8-10]. Besides other orthobunyaviruses, e.g. Oropouche virus or Akabane virus (AKAV), various members of the genus *Orbivirus* within the family *Reoviridae* have been isolated from Culicoides biting midges. As an example,

bluetongue virus (BTV) is transmitted between its ruminant hosts mainly by the bites of midges [11]. European experience showed that vaccination of livestock against BTV has had a major role in reducing the virus circulation and even in eradicating the disease from some areas [12] and [13]. Based on similarities between BTV and SBV regarding involved insect vectors and affected host species the same is suspected for SBV. In addition, inactivated vaccines against Akabane virus and Aino virus, which are members of the Simbu serogroup within the genus *Orthobunyavirus*, can prevent the diseases [14]. In all likelihood, vaccination against SBV will be one of the most important aspects of disease control on the farm as well as on a region or country level. In the present study, several inactivated vaccine formulations have been produced and subsequently tested in sheep and cattle regarding their ability to induce neutralising antibodies and to prevent viraemia after experimental challenge infection.

2. Materials and methods

2.1 Vaccines

In order to test the immunogenicity of viruses grown on different cell substrates, five different prototype vaccine formulations were produced (Table 1); all of them were inactivated SBV preparations in aqueous solution. SBV was either grown on two different baby hamster kidney (BHK-21) cell lines (vaccines "BHKCT-HT", "BHK13-HT", "BHK13-LT") or on MA-104 cells (vaccines "MA-HT" and "MA-LT"), a monkey kidney cell line. Both cell substrates, BHK-21 as well as MA-104 support the growth of the virus to high titers. The antigen was quantitated using the infectious titre of SBV before inactivation with binary ethylenimine (BEI), using either a long (using 10 mM of BEI for 72 hours at 37°C) or a short (using 2 mM of BEI for 12 hours at 37°C) protocol. Vaccine candidates contained antigen concentration as follows: 6.1 log10 50% tissue culture infectious doses per ml ($TCID_{50}$/ml) (MA-HT) or 5.7 log10 $TCID_{50}$/ml (BHKCT-HT, BHK13-HT, MA-LT) or 4.7 log10 $TCID_{50}$/ml (BHK13-LT). Saponin (0,125 µg per 1ml) and aluminium hydroxide (6,65mg per ml) were used as adjuvants in all vaccine candidate formulations and the pH values were adjusted to pH 6.8-7.2 at 20° C. All candidate vaccines were tested for the absence of bacterial contamination and for successful virus inactivation by two subsequent passages on BHK-21 cells. The vaccines were kept at 4°C until use.

Table 1: Vaccines and animal groups.

Vaccines				Animals	
Name	**Cell line**	**Infectious titre used**	**Inactivation**	**Animal group**	**Animal number**
BHKCT-HT	BHK-21 clone CT	5.7 log10 $TCID_{50}$/ml	long protocol	A (sheep) G (cattle)	S01 - S05 C01 - C06
BHK13-HT	BHK-21 clone 13	5.7 log10 $TCID_{50}$/ml	short protocol	B (sheep)	S06 - S10
BHK13-LT	BHK-21 clone 13	4.7 log10 $TCID_{50}$/ml	short protocol	C (sheep)	S11 - S15
MA-HT	MA-104	6.1 log10 $TCID_{50}$/ml	short protocol	D (sheep) H (cattle)	S16 - S20 C07 - C10
MA-LT	MA-104	5.7 log10 $TCID_{50}$/ml	long protocol	E (sheep) I (cattle)	S21 - S25 C11 - C16
unvaccinated control				F (sheep) K (cattle)	S26 - S30 C17 - C22

2.2. Animals

The experimental protocol was reviewed by a state ethics commission and has been approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Vorpommern, Rostock, Germany, ref. LALLF M-V TSD/7221.3-1.1-004/12).

Twenty-five SBV-naive sheep of European domestic breeds (7 – 9 months of age) were assigned to 5 groups of 5 animals each, which were immunised subcutaneously with one of the prototype vaccines (see Table 1). Another 5 sheep were kept as unvaccinated controls. Male and female animals were distributed equally.

In addition, 22 SBV antibody-negative female Holstein-Friesian cattle were assigned to four groups of four (group H) or six animals (groups G, I and K) each. Animals in group G, H and I were immunised subcutaneously with vaccines BHKCT-HT, MA-HT and MA-LT, respectively. Cattle in group K were kept as unvaccinated controls. On the day of the first vaccination, the animals were between 8 and 12 months of age.

In each case, the animals were vaccinated twice three weeks apart and three weeks after the second vaccination both vaccinated and control animals were inoculated with 2 x 0.5ml of an SBV field strain that was only passaged in the natural host [15]. During the entire study, rectal body temperatures were measured daily, and the animals were examined for clinical signs by veterinarians.

2.3. Sampling, real-time RT-PCR and serology

Following the first vaccination, serum samples were collected at days 0, 3, 4, 7 and weekly thereafter. After the second vaccination, serum samples were taken in weekly intervals. Following challenge infection, serum samples were taken daily during the first eight days and on days 14 and 21. Samples of spleen, tonsils, and mesenteric and mandibular lymph nodes were taken at autopsy on days 22 - 29 after challenge infection and homogenized in 1 ml MEM.

RNA from serum and tissue samples taken at autopsy was extracted using the MagAttract Virus Mini M48 Kit for automated extraction (Qiagen, Germany) according to the manufacturer's recommendations. SBV genome loads were determined by a reverse transcription real-time PCR (RT-qPCR) [16] with an external standard based on the S genome segment. Furthermore, serum samples were analyzed with a commercially available SBV antibody ELISA (ID Screen® Schmallenberg virus Indirect, IDvet, France) using the recommended cut-off of 70% relative optical density compared to the positive control, and in a standard micro-neutralisation assay as described previously [17].

3. Results

3.1 Clinical observations and post-mortem examinations

Following the first vaccination with the vaccine prototypes no adverse side effects were observed; none of the animals showed fever or any other clinical sign. After the second vaccination one cow immunised with vaccine MA-HT (group H) developed a low-grade swelling at the injection site for 2 days.

After the challenge infection, one unvaccinated cow developed fever on day 3, another one showed mild diarrhoea for three days. One animal from group I had nasal discharge for one day.

Autopsy of the animals did not reveal any significant gross lesions. The mesenteric lymph nodes of all but one (S30) unvaccinated animals were PCR-positive; on average 2.86E+03 genome copies per mg (copies/mg) were detected. In addition, SBV RNA was found in the mandibular lymph nodes of 3 out of 5 unvaccinated sheep (S27 – S29) and of all control cattle (average 2.68E+01 copies/mg), the tonsils of S27 – S29 and C18 – C20 (average 9.90E+01copies/mg), and spleens of 4 out of 5 unvaccinated sheep (S26 – S29; average 4.57E+03 copies/mg) and of two control calves (C17, C21; average 1.40E+01 copies/mg). No viral RNA was detected in any of the vaccinated animals.

3.1.1. Antibody response

On the day of the first vaccination, all animals were negative in both serological assays (data not shown). Before challenge infection, no antibodies could be detected in the unvaccinated animals. Three weeks after infection all but one (S30) control sheep and cattle scored positive in the neutralisation assay. Antibodies were found in cattle and in 2 out of the 5 unvaccinated sheep (S26, S29) by ELISA as well. Despite an increasing sample OD relative to the positive control OD value (S/P) both the control sheep S27 and S28 scored negative in the ELISA (Fig. 1).

Three weeks after the first immunisation with vaccine BHKCT-HT, BHK13-HT or BHK13-LT (SBV grown on BHK cells), all sheep and cattle were negative in the ELISA, while in S07, S08, S10 (BHKCT-HT), and S04 (BHK13-HT) low antibody titres were detected in the neutralisation assay. Following the second vaccination antibodies were detected in at least one serological assay, in most cases a considerable increase of neutralising antibodies was seen. Three weeks after challenge infection 8 out of 15 sheep (S04, S06, S07, S09, S10, S11, S12, S15) and 5 out of 6 cattle (C01 – C05) were positive in both assays, 7 sheep (S01 - S03, S05, S08, S13, S14) and the remaining cattle (C6) were positive in the neutralisation test only, and S15 in the ELISA assay only (see Fig. 1).

After one immunisation with vaccines MA-HT or MA-LT (SBV grown on MA-104 cells), all cattle and all but two sheep scored negative in both serological assays. S22 and S23 had titres of 1:5 and 1:7, respectively, in the neutralisation assay. Following the second vaccination, in S19, S24, C08, and C14 no antibodies could be detected. S16, S21, C07, C09, and C10 scored positive in both serological assays, while the remaining animals were positive in the neutralisation assay only. Three weeks after challenge infection all sheep of group D and 4 out of 5 sheep of group E were positive in the neutralisation assay, in animal S16 antibodies could be detected by the ELISA, and animal S24 was negative in both assays. In all cattle of group H (high titre of SBV) antibodies were detectable by ELISA and neutralisation assay. The same is true for C12 and C13 (group I, low SBV titre), C11, C15, and C16 scored positive only in the neutralisation assay, and in C14 no antibodies could be detected by any test (Fig. 1).

After the second immunisation an increase of the average neutralising antibody titres was observed, while after challenge infection, most of the neutralisation titres remained constant in all vaccinated groups.

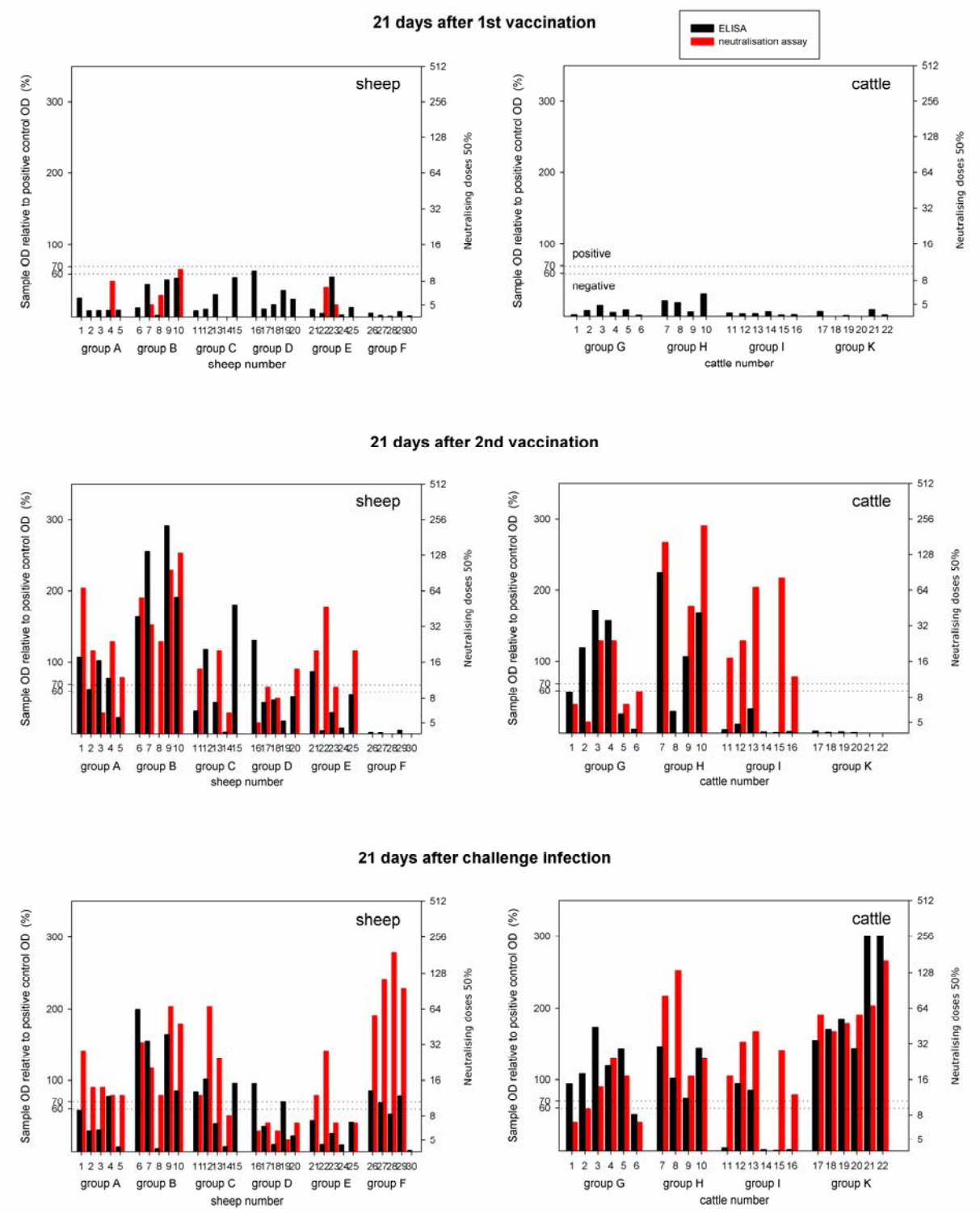

Figure 1:

Results of an SBV antibody ELISA (black bar) and a neutralisation assay (red bar). The cut-off values of the antibody ELISA are marked by a dotted line.

3.1.2. Real-time RT-PCR

Following the first vaccination SBV genome was not detected in any animal (data not shown), confirming the successful inactivation of SBV with both the short and long BEI inactivation procedure.

After challenge infection, all but one (S30) unvaccinated sheep scored positive in the RT-qPCR between day 2 and 4 (S27 - S29) or 5 (S26). In 1 out of 6 unvaccinated cattle (C19) SBV-genome was first detectable on day 1 after infection, the other 5 calves scored positive on day 2 for the first time. SBV genome remained detectable until day 5 (C17, C19 - C21), 6 (C22) or 7 (C18). Three out of 6 cattle immunised with vaccine MA-LT (C12, C13, C16) were positive in the RT-qPCR on day 3 (Fig. 2), while the animals vaccinated with MA-HT vaccines did not develop RNAemia (RNA in the blood) upon challenge.

In serum samples taken from all vaccinated sheep, from control sheep S30, and from all cattle of groups G and H (high titre vaccine groups), viral RNA could not be detected following challenge infection (Fig. 2).

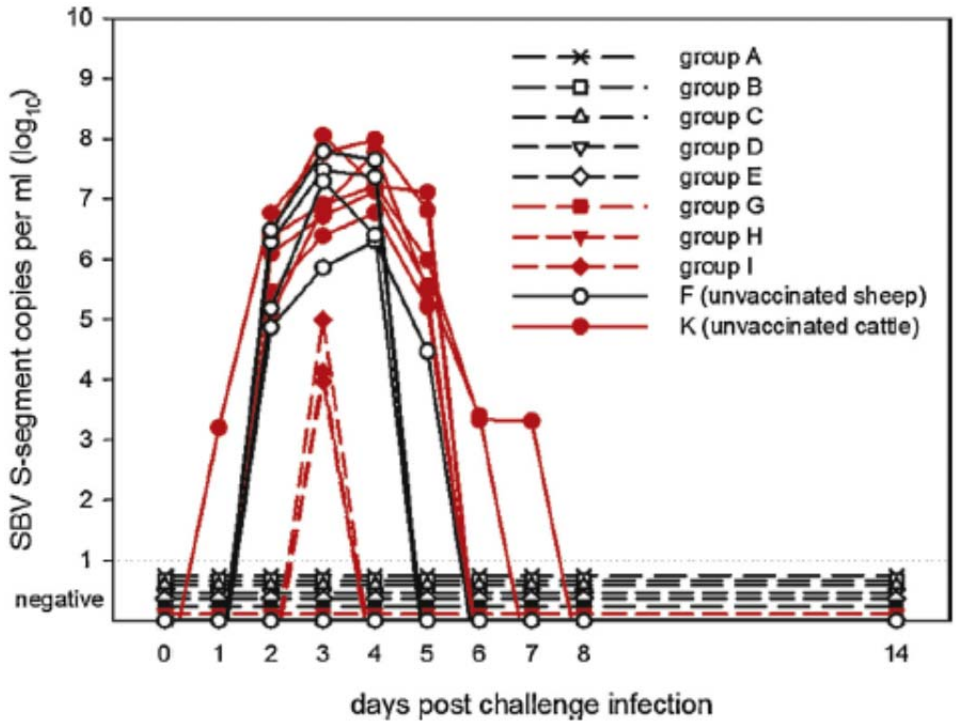

Figure 2:

RT-qPCR results of serum samples taken after challenge infection. Sheep groups are depicted in black with unfilled symbols, cattle groups in red with filled symbols. Graphs showing the same symbol belong to animals immunised with an identical vaccine.

4. Discussion

Since its emergence in late 2011 near the German-Dutch border, SBV spread very rapidly to other European countries. The virus is responsible for a short febrile phase, diarrhoea and a reduction in milk yield in adult ruminants, but above all for severe foetal malformation and stillbirth, which causes considerable economic loss [18] and [19]. As other related Simbu serogroup viruses, e.g. AKAV or Aino virus, causes similar clinical signs [20], [21] and [22] and vaccines against AKAV induce neutralising antibodies that protects from clinical disease [23], vaccination against SBV could play an important role in disease control. This assumption is supported by the latest experience with insect-transmitted viruses in northern Europe. After the emergence of BTV, livestock was vaccinated at a high percentage. In regions with a high level of vaccine coverage up to 80% - 90%, virus circulation was significantly reduced, from some areas even eradicated [12], [24], [25] and [26]. Due to similarities between BTV and SBV regarding host species as well as involved vectors, the same may be considered for SBV. Although a high within-herd seroprevalence of antibodies against SBV exists in cows and ewes exposed to infection in 2011 [27] and [28], seroprevalence significantly increased with age of the animals [29]. Mating of those seronegative animals in the following vector season may result in new SBV-infections during a critical period of pregnancy, which could be prevented by vaccination prior to first gestation. Furthermore, regions which are yet free of SBV could protect their livestock from damages and side-effects induced by SBV-infection, including trade restrictions.

Inactivated vaccines generally show a high safety profile and can be developed within a relatively short timeframe making them an ideal approach as first vaccine candidate against newly emerging pathogens. We developed five different inactivated prototype vaccines and tested them in cattle and sheep. All test-vaccines were formulated with Al(OH)3 and saponin, an adjuvant combination which is described to modulate the cell mediated immune system and enhances antibody production [30] and [31]. In our experiments none of the animals showed significant adverse effects. Furthermore, most of the animals developed detectable neutralizing SBV antibodies levels upon vaccination (Fig. 2). Importantly, upon challenge infection, RNAemia was completely prevented by four prototype vaccines and considerably reduced by the fifth. Those data suggest that protection from virus infection is only partially mediated by neutralizing antibodies and additional still undetermined mechanisms, most likely associated with cellular immunity, contributed to virus clearance upon SBV challenge. The presence of viral RNA in the lymphoid organs, which has been previously reported after SBV experimental infection [15] and [17], was tested in addition to SBV-RNA screening of

serum. In contrast to the unvaccinated controls, all vaccinated animals were clearly negative for SBV-RNA in the lymphoid organs at the time of autopsy. Interestingly, one of the unvaccinated control sheep showed neither RNAemia, nor RT-qPCR-positive tissue samples, nor seroconversion after challenge infection, the reason for that observation remains unclear. Possible explanations are a failed injection or a status of natural resistance to SBV infection.

Whether prevention of foetal malformation could be achieved by immunisation of the dam against SBV, may be inferred from closely related viruses. Pregnant goats for instance showed neither clinical signs nor viraemia after immunisation against AKAV and subsequent challenge, and the foetuses were virologically negative as well [23]. However, further studies will be necessary to verify this assumption in the case of SBV and to proof foetal protection upon vaccination. Nevertheless, the absence of detectable RNA in most vaccine groups is very promising and it is highly unlikely that challenge virus could be transmitted to the foetus if even no viral genomes can be detected in the serum.

Although RNAemia was prevented or markedly reduced by vaccination, antibodies were not detected in every animal prior to challenge infection in every test. In contrast to the previously reported correlation of >98% between ELISA (ID Screen® Schmallenberg virus Indirect) and neutralisation test [32], in this study a higher rate of samples was positive in the neutralisation test than in the ELISA.

A very interesting result of this study was that the highest neutralising antibody titres were found in non-vaccinated sheep after challenge infection. A similar observation was already made in a previous study testing Rift Valley fever (RVF) vaccines; after immunisation of sheep with different RVF vaccines and subsequent challenge infection, the control animals also showed higher neutralising titres as the partially protected vaccinees [33]. However, the SBV vaccine prototypes characterized in this study prevented RNAemia in sheep completely despite a low level of neutralising antibodies.

In our study, the titre of neutralising antibodies was dependent on the production cell line and the viral titre prior to inactivation. A dose dependence of the cell culture supernatant used for vaccine preparation was described for AKAV as well, independent whether inactivated or attenuated live vaccines were used [23] and [34]. At least 10^5.5 $TCID_{50}$/ml of virus were reported to be necessary for vaccine development. As 2 ml of a vaccine containing 6.1 log 10 $TCID_{50}$/ml virus grown on MA-104 cells prevented RNAemia completely, but in half of the calves which were immunized with 5.7 log 10 $TCID_{50}$/ml viral genome was detectable for one day, a similar minimal dose may be assumed for SBV. However, in vaccines produced on BHK-21-cells, the lower viral titre (5.7 log 10 TCID50/ml) prevented RNAemia completely

in both animal species, in sheep merely 4.7 log 10 TCID50/ml were necessary. The differences found might be explainable by a different immunogenicity of the vaccine candidates depending on the cell line the virus was grown. However, it is more likely that different amounts of antigen were used for vaccination. Considering that the live virus was titrated and these data were used for the preparation of the vaccines, the absolute quantity of SBV-antigen present in the different prototype vaccines after inactivation was not determined precisely. An SBV potency assay which was not available at the time when the study was conducted will be used for the exact quantification of SBV antigen in the final vaccine formulation in the future. In addition, a further explanation for the differences in efficacy may be the inactivation protocol itself. Dependence of the immunogenicity from the system of virus preparation (chick embryos or different cell lines) before inactivation was also seen for Rift Valley fever virus, another bunyavirus (reviewed in [35]).The reasons for these observations are still unclear.

In summary, in this proof of concept study we could demonstrate that killed vaccine candidates against SBV, produced with a technology applicable to commercial vaccine manufacturing are safe and protect cattle and sheep from challenge virus infection. Parameters like the duration and onset of immunity upon vaccination as well as vaccine efficacy after single immunisation and vaccine safety in pregnant animals will be topic of further studies. We conclude that a killed vaccine as described here has desirable properties, and our data suggest that it could be successfully applied to support efforts for controlling SBV spread as well as disease prevention in domestic ruminants.

Competing interests

Veljko M. Nikolin is an employee of Boehringer Ingelheim Veterinary Research Center GmbH&CoKG; this does not alter the adherence to the *Vaccine* policies on sharing data.

Role of the funding source

The funders manufactured the vaccines used in the present study and were involved in the study design. The funders played no role in collection, analysis, and interpretation of data.

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Single immunization with an inactivated vaccine protects sheep from Schmallenberg virus infection

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Abstract

The arthropod-borne Schmallenberg virus (SBV), family *Orthobunyaviridae*, emerged in Europe in 2011. SBV is associated with a mild disease in adult ruminants but fetal malformation after an infection during a critical phase of pregnancy. A number of inactivated vaccines have been developed; their efficacy after two injections was demonstrated. To make the vaccination of sheep more efficient and economic the effect of a single immunization with one of these vaccines was investigated in the present study. Five vaccinated sheep and five additional control sheep were inoculated with SBV three weeks after vaccination and the results of a competitive ELISA, a standard microneutralization test and an SBV-specific real-time RT-PCR confirmed vaccine efficacy by demonstrating complete inhibition of viral replication in immunized animals.

Introduction, Methods and Results

A previously unknown pathogen from the family *Orthobunyaviridae* emerged in Europe in autumn 2011 and was named Schmallenberg virus (SBV) according to the location of its discovery [1]. Midges (Culicoides spp.) are involved in its transmission [2-4]. While disease was first observed in cattle, sheep and goats, infection has also been detected in deer, bison, alpaca, moose and other wild ruminants [5]. The clinical picture is characterized by mild febrile disease in adult ruminants and the potential development of fetal malformations after transplacental infection [6-9]. An SBV-infection can be confirmed through detection of viral RNA both in serum during the first week post infection and in tissue samples [10].

As an effective instrument for disease control different inactivated vaccines have been developed and tested [11]. Besides, two commercial inactivated vaccines have already been granted a provisional marketing authorization in the United Kingdom and France, respectively [12, 13].

Until now, only studies about a protective effect after two vaccinations have been published. Reduction to a single injection minimizes workload and costs, which is especially important for sheep owners, as the animals are usually individually caught and restrained on the pastures for every injection. Therefore, the influence of a single immunization on a subsequent SBV-inoculation of sheep was investigated in the present study.

Five SBV-negative yearling sheep (S01 to S05) of European domestic breeds received a single subcutaneous injection with 2 ml of the MA-HT prototype vaccine from a previous study [11]. Five additional control sheep (S06 to S10) were left unvaccinated.

Three weeks after vaccination all animals were inoculated with 2 x 0.5ml of calf serum containing an SBV field strain that was only passaged in the natural host. The production of this infectious serum has been described earlier [10]. The serological status was monitored weekly by a blocking ELISA (ID Screen® Schmallenberg virus Competition, ID vet, France) and a standard microneutralization test (SNT) [14]. Additionally, blood samples were taken daily on the 8 days following challenge infection and tested by ELISA and an SBV-specific reverse transcription real-time PCR (RT-qPCR) including an external standard based on the small (S) genome segment [15]. Rectal body temperatures were recorded daily during the entire study and the animals were examined daily for clinical signs. Autopsy was conducted three weeks after challenge infection and samples of spleen, mesenteric and mandibular lymph node and tonsils were taken and tested by RT-qPCR.

The experimental protocol was reviewed by a state ethics commission and has been approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Vorpommern, Rostock, Germany. Ref. No. LALLF M-V TSD/7221.3-1.1-004/12).

The SBV-antibody-ELISA was used according to the manufacturer's instructions. Results were calculated as the ratio of the optical density (OD) of the sample and the OD of the negative control (S/N, %). Samples with an S/N-value of 40% or less were considered positive. SNT-titers were calculated as reciprocal of the serum dilution showing 50% virus neutralization (neutralizing dose 50, ND50). Titers of 5 or more were considered positive. The MagAttract Virus Mini M48 Kit (Qiagen. Germany) was used to extract nucleic acid from serum and tissue samples according to the manufacturer's recommendations.

All animals were serologically and virologically negative for SBV on the day of immunization. Vaccination was not associated with adverse side effects. None of the animals showed clinical signs of disease after challenge infection; the rectal body temperature remained within a normal range and autopsy did not reveal any gross pathological lesions.

On the day of challenge infection all five control animals were negative for SBV in both serological tests (Table 1). First SBV-specific antibodies were detected in controls on day 7 after challenge by SNT and on day 8 after challenge by ELISA. Neutralizing titers rose to values between 33 and 266 ND50 until day 21 post infection (Table 1).

All vaccinated animals were positive for SBV prior to challenge infection in at least one serological test (Table 1) and, thereafter, neutralizing titers remained largely constant with values between 7 and 14 ND50 on day 21 post challenge. The first antibodies were detected by SNT in S02 on day 7 after vaccination while the ELISA gave negative results for this animal for all sampling dates except day 14 after vaccination (Table 1). Samples of S01, S03 and S05 gave positive results in the SNT starting from day 14 after vaccination while S01 and S03 scored negative in the ELISA throughout the study. S05 gave only one doubtful ELISA result on day 14 post vaccination. In S04 neutralizing antibodies were detectable only one week after challenge infection but it scored positive in the ELISA on day 14 after vaccination and doubtful on the day of challenge infection.

animal	group	SNT						ELISA						qRT-PCR
		0 dpv	14 dpv	0 dpc	7 dpc	14 dpc	21 dpc	0 dpv	14 dpv	0 dpc	7 dpc	14 dpc	21 dpc	tissue
S01	vac	<5	**17**	**12**	**10**	**10**	**7**	91.6	51.5	53.4	59.6	56.5	53.5	-
S02	vac	<5	**56**	**24**	**14**	**10**	**14**	92.0	**45.8**	54.9	55.1	65.6	70.2	-
S03	vac	<5	**12**	**7**	**6**	**7**	**14**	97.1	50.8	60.1	64.7	51.3	50.1	-
S04	vac	<5	<5	<5	**7**	**7**	**7**	84.4	**33.0**	**42.4**	**46.0**	51.8	56.6	-
S05	vac	<5	**20**	**28**	**14**	**12**	**12**	92.8	**47.5**	54.7	57.3	54.6	88.4	-
S06	co	ND	ND	<5	**14**	**67**	**40**	ND	ND	92.5	**42.5**	**26.9**	**26.5**	+
S07	co	<5	<5	<5	**12**	**375**	**266**	94.9	89.6	94.8	**43.8**	**14.6**	**17.5**	+
S08	co	<5	<5	<5	**8**	**160**	**67**	95.9	86.7	92.0	**30.2**	**30.6**	**37.9**	+
S09	co	<5	<5	<5	**10**	**224**	**160**	99.6	91.0	97.3	**44.5**	**23.1**	**21.9**	+
S10	co	<5	<5	<5	**8**	**56**	**33**	82.4	67.9	73.9	58.8	**32.3**	**27.6**	+

Table 1: Key serological results for vaccinated (vac) and control (co) animals are presented. Serological results are given for 0 and 14 days post vaccination (dpv) to 21 days post challenge infection (dpc). Neutralizing titers (SNT) are given as the reciprocal of the serum dilution showing 50% virus neutralization. Titers of 5 or more were considered positive. ELISA results are calculated as the ratio of the optical density (OD) of the sample and the OD of the negative control (S/N, %). Samples with an S/N value of 40% or less are considered positive. Positive or doubtful ELISA results and positive SNT results are highlighted by bold figures. Results for RNA detection are given for tissue samples obtained at autopsy.

After challenge infection, SBV-RNA was detectable in serum samples of all control animals for at least 3 consecutive days (Table 2). The mean maximum genome load in serum samples was $9.2x10^4$ genome copies per ml. Most tissue samples of the control animals gave positive PCR results as well. Only tonsils and mesenteric lymph nodes of S10, and mandibular lymph nodes of S06 scored negative. Mean genome loads per gram organ weight were $1.2x10^4$ copies/g for mandibular lymph nodes (minimum value: $4.9x10^2$ copies/g; maximum value: $3.6x10^4$ copies/g), $8.7x10^4$ copies/g for mesenteric lymph nodes (min: $5.9x10^1$; max: $3.4x10^5$), $1.2x10^5$ copies/g for spleens (min: $6.8x10^3$; max: $3.9x10^5$) and $1.6x10^5$ copies/g for tonsils (min: $4{,}7x10^1$; max: $6.0x10^5$).

In contrast, viral RNA was not detected in any serum or tissue sample from the vaccinated animals.

animal	group	number of SBV RNA copies per ml serum								
		0 dpc	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc	8 dpc
S 06	co	-	-	$4{,}5 \times 10^4$	$3{,}9 \times 10^3$	$2{,}2 \times 10^3$	ND	-	-	-
S 07	co	-	-	$1{,}9 \times 10^3$	$1{,}2 \times 10^4$	$7{,}0 \times 10^5$	ND	$1{,}4 \times 10^3$	-	-
S 08	co	-	-	$1{,}4 \times 10^4$	$1{,}5 \times 10^4$	$2{,}5 \times 10^4$	ND	-	-	-
S 09	co	-	-	$5{,}2 \times 10^2$	$3{,}3 \times 10^4$	$1{,}5 \times 10^5$	ND	-	-	-
S 10	co	-	-	$8{,}5 \times 10^2$	$1{,}8 \times 10^3$	$6{,}4 \times 10^3$	ND	-	-	-

Table 2: RNA detection in serum post challenge.

Results are given for 0 to 8 days post challenge (dpc) for control animals (co). Dashes represent negative PCR results of the respective samples. Viral RNA was not detected in the serum of vaccinated animals at any time. Therefore, vaccinated animals were not included in the table. From 5 dpc serum samples were not available. Consequently, the RNA load could not be determined (ND) for this time point.

Discussion

In the unvaccinated control animals PCR results demonstrated viral replication and dissemination. The serological results support this observation as the SBV-infection induced a pronounced humoral immune response. In all vaccinated sheep, on the other hand, the absence of RNAemia demonstrates the protective effect of immunization. Furthermore, the antibody titers remained constant which suggests that the virus is eliminated before a memory immune response with an antibody boost could be triggered. The latter is in accordance with results of an earlier study in cattle [14] during which constant neutralizing titers were detected in seropositive animals after a second experimental SBV-infection. Interestingly, the single shot vaccination was highly efficacious and could even prevent both viremia and infection of target tissues such as mesenteric lymphnodes.

Interestingly, neutralizing titers are very low in vaccinated animals in this study and only a few ELISA results of their serum samples exceed the cut-off value. Thus, further factors, e.g. a cellular immune response, may contribute to the protective effect of vaccination. Similar observations have been reported for Rift Valley Fever virus (RVFV, family *Bunyaviridae*, genus Phlebovirus). Neutralizing antibodies are primarily responsible for protection against RVFV-infection [16]. However, one of six lambs treated with an inactivated vaccine showed a reduction in viremia and lack of clinical symptoms although detectable neutralizing antibodies were missing at the time of infection [17]. Furthermore, a study on Crimean-Congo hemorrhagic fever virus (CCHFV, family *Bunyaviridae*, genus Nairovirus) reports that an inactivated vaccine is able to elicit a considerable T-cell reaction in humans as measured by IFN-gamma production [18].

Unfortunately, there are no immunological studies available which deal with orthobunya virus vaccines. Thus, the exact mechanism underlying our observations remains unclear. However, as saponins are able to stimulate cellular immune responses [19], this adjuvant used for the formulation of the vaccine may be an important factor for vaccine efficacy and protection from SBV replication post challenge infection.

In conclusion, the present study demonstrated the complete protection of sheep from SBV-infection after a single injection while the underlying immunological mechanism needs to be further investigated.

Competing interests

The authors have no any financial, personal, or professional interests that could be construed to have influenced this paper.

Authors' contributions

Conceived and designed the experiments: KW, MB. Performed the experiments: SH, KW. Analyzed the data: SH, KW. Wrote the paper: SH, KW, MB. All authors read and approved the final manuscript.

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4 Discussion

4.1 Environmental factors influencing SBV circulation

After the first main SBV-epidemic in central Europe, European Veterinary Administrations and politicians as well as farmers had to make decisions on how to deal with this pathogen in the future. The application of available control measures – among which vaccination is an important option - has to be weighed against potential financial losses caused by new disease outbreaks and restrictions imposed by trading partners (European Food Safety Authority 2014). In order to devise effective strategies for minimizing future SBV-impact it is further necessary to take into account epidemiological and biological characteristics of this virus. Some influencing factors are illustrated in the following.

Climate has proven to be a decisive factor for AKAV-vector activity and virus circulation on the Australian continent. Yearly peaks in vector activity occur in coastal regions during the warm and wet season, which means especially the Australian spring, summer and autumn (September to April). Increased vector activity subsequently leads to increased virus transmission. Immunologically naïve sentinel cattle are reliably infected with AKAV during the period of increased vector activity. Beyond that, the area of virus circulation may expand in certain years if climatic factors are particularly favorable for midge vectors (Animal Health Australia 2001, Animal Health Australia 2013).

In Europe local geographical and climatic factors seem to influence SBV-dissemination in a similar way. For example, it could be observed that virus circulation in rather large countries like the UK, France or Germany was inhomogeneous as a majority of susceptible hosts remained naïve in some areas while high sero-prevalences had already been reached in other subpopulations (Anonymous 2012, Conraths, Kamer et al. 2013, Gache, Dominguez et al. 2013, Valas, Baudry et al. 2014). It has also been proposed, that a general drought in north-western Europe during 2011 might have favored the swift spread of SBV (Calzolari and Albieri 2013). Furthermore, SBV-transmission has been demonstrated to be significantly reduced during central European winter as compared to summer or autumn months (Wernike, Kohn et al. 2013, Wernike, Silaghi et al. 2014). This can be explained by reduced vector activity and inhibition of viral replication within the insect due to low temperature (Mellor, Boorman et al. 2000). In northern Europe climatic conditions might play an even more important role with geographical distances contributing to the observed pattern of spread. In Sweden and Norway, for example, a gradient in SBV-specific sero-prevalence rates could be detected at the end of 2012 with high values in the south and declining prevalences in northward direction (Chenais, Stahl et al. 2013, Wisløff, Nordvik et al. 2014). This fact could on the one hand be explained by sheer distance: SBV was introduced in the southmost parts of Sweden (Chenais, Stahl et al. 2013) and seroprevalences after the first vector season were low in regions which were distant from the point of introduction. On the other hand northward spread of the virus is restricted by the limits of vector distribution (Ander, Meiswinkel et al. 2012). Thus, SBV-transmission in regions located further north could have been impaired by reduced numbers of available vectors and by slower virus propagation in infected insects due

to lower average temperatures. Finally, lower livestock population density in northern parts of the country could have been a further influencing factor.
Another example can be found in Scotland which reported its first affected calf in April 2013 compared to the first SBV-infected lambs in England which were found already in January 2012. It has been shown in a statistical model that this discrepancy can indeed be explained by differences in climatic conditions (Bessell, Searle et al. 2013). However, it cannot be excluded that Scotland's distance from the point of the first introduction (in southern England) plays a role as well.
In conclusion, application of control measures would be of special importance in regions which enable the occurrence of abundant vector populations. Unfortunately, current knowledge about the biology of the identified vectors and potential reservoirs in Europe is not sufficient to enable detailed predictions on virus prevalence and persistence in a given area. E.g. the mechanism which enables SBV to overwinter in central Europe has not been elucidated yet, although vertical transmission in midges could be a possibility (Larska, Lechowski et al. 2013). Published scientific results are contradictory in this respect, however (De Regge, Madder et al. 2014). Thus, this kind of data allows only for a rather rough estimation of the probability of disease occurrence in a given area.

A more precise prediction can be made if host immunology is considered. It has to be kept in mind however, that continuous collection of serological data from the field is necessary for an adequate risk assessment. Experimental results demonstrate that SBV-specific antibody titers originating from primary infection inhibit viral replication after reinfection (Wernike, Eschbaumer et al. 2013, Rodriguez-Prieto, Kukielka et al. 2014). Antibody titers in adult animals have furthermore been shown to persist for at least two years (Elbers, Stockhofe-Zurwieden et al. 2014). However, a small proportion of susceptible animals remains even in highly affected livestock populations (Elbers, Loeffen et al. 2012, Méroc, Poskin et al. 2013). This is all the more true in less affected regions (Chenais, Stahl et al. 2013, Gache, Dominguez et al. 2013). Moreover, in many countries sero-prevalences in small ruminants have been found to be significantly lower than in cattle (Helmer, Eibach et al. 2013, Méroc, De Regge et al. 2013, Valas, Baudry et al. 2014, Wernike, Conraths et al. 2014) and wild ruminants further add to the number of available hosts (Claine, Coupeau et al. 2013, Tarlinton, Daly et al. 2013). Remarkably, active virus circulation has been shown to persist under these circumstances even in highly affected areas although on a decreased level (Claine, Coupeau et al. 2013, Méroc, Poskin et al. 2013, De Regge, Madder et al. 2014). This is in accordance with observations of AKAV-circulation in Australia which demonstrate that virus circulation in the vector population must be expected even against the background of a majority of immune host animals (Animal Health Australia 2013). Another aspect which has been observed is that sero-prevalence levels appear to decrease over time (Méroc, Poskin et al. 2013). Although this is not necessarily synonymous with loss of immune protection, reinfection of formerly immune animals might be possible. Even more important: The number of immune animals in the livestock population is continually reduced by slaughter while naïve offspring is used for restocking. In consequence, herd immunity is increasingly impaired. In summary, control strategies ought to aim at protecting naïve individuals from infection as best

as possible. Even more effective would be to reduce the number of susceptible animals. This could be either by natural infection at an early age or by vaccination.

In general, concerted efforts for SBV-control will have to be made on a European level – and beyond - as the entirety of all the factors described above will potentially sustain viral circulation over prolonged periods and lead to recurring disease outbreaks as reintroduction from areas with active virus circulation may occur into regions where natural immunity is waning. Given the velocity of spread which SBV has demonstrated, this may not only happen on a national level but also across large parts of the continent. Japan may be cited as an example for such an epidemiological scenario as Akabane disease outbreaks continue to occur regularly there (Yamakawa, Yanase et al. 2006, Oem, Yoon et al. 2012) and incursions of AKAV-infected vectors seem to be a major cause of these outbreaks (Yamakawa, Yanase et al. 2006, Kono, Hirata et al. 2008).

4.2 Strategies for SBV-control

As mentioned before, control of arthropod borne diseases may be attempted in a number of ways (Carpenter, Mellor et al. 2008) and preventing the contact between vector and host is an essential component of many strategies. Reducing the abundance of potential vectors in an area by destruction of their biotopes and/or application of pesticides could be one option. However this is usually associated with detrimental ecological effects. Beside that, accumulations of stagnant water and moist organic matter which regularly occur on farms may serve as breeding sites for some midge species (Zimmer, Smeets et al. 2013) and the chance of successfully reducing such artificial habitats has to be judged critically (Harrup, Gubbins et al. 2014).

Another option is treatment of livestock with insecticidal products. A variety of such products is available today. They are intended for application via pour-on treatment, dip-washing or injection. Products for pour-on application have been shown to reduce feeding activity of midges on the respective host animal (Weiher, Bauer et al. 2014) and effectively knock down feeding insects (Mehlhorn, Schmahl et al. 2008, Schmahl, Klimpel et al. 2009), but there is no proof that insecticide treatment of livestock is able to reduce SBV-transmission (Helmer, Eibach et al. 2013, Veldhuis, Carp-van Dijken et al. 2014). Insufficient treatment intervals are probably an important factor as the drug effect persists only for a limited time of a few weeks (Standfast, Muller et al. 1985, Mehlhorn, Schmahl et al. 2008, Schmahl, Klimpel et al. 2009, Weiher, Bauer et al. 2014). Thus, for optimal effects application has to be repeated regularly which is quite laborious.

Another, potentially very effective method of protection is housing. Importantly, the effectiveness of this approach is highly dependent on stable design. E.g. windows, doors or other openings should be closed or equipped with adequate mesh constructions, which can further be impregnated with insecticides. Unfortunately, constructions which offer optimal protection are costly. If prevailing housing systems are used, which are not able to prevent intrusion of midges completely, infection can not be entirely prevented (Veldhuis, Carp-van

Dijken et al. 2014) as Culicoides midges of the species C. obsoletus, C. scoticus, C. dewulfii and C. chiopterus have been shown to enter stables readily and feed on housed livestock especially when outside temperatures are low (Baldet, Delecolle et al. 2008, Baylis, Parkin et al. 2010, Viennet, Garros et al. 2012). However, this kind of housing seems to have some effect for small ruminants (Helmer, Eibach et al. 2013).

Another drawback of the approach in general is that housing of animals over longer periods is not always wanted. In the case of SBV, for example, it would be necessary to house female animals at least during the period of maximum fetal susceptibility in order to significantly reduce incidence of fetal SBV-related disease. While this would be only about one or two months for sheep it correlates with at least 4 months for cattle if the corresponding data for AKAV is considered (Kurogi, Inaba et al. 1977, Hashiguchi, Nanba et al. 1979, Kirkland, Barry et al. 1988).

Analyses of factors influencing the risk for SBV-infection in small ruminants have confirmed some protective effect of mating outside the periods of maximum vector activity (Luttikholt, Veldhuis et al. 2014). In Australia this strategy is successfully applied, both for small ruminants and for cattle, to reduce the occurrence of Akabane disease within the endemic area (Animal Health Australia 2001). In this way disease outbreaks occur only at irregular intervals on the fringes of the endemic zone if insect vectors are able to extend their area of distribution during years with favorable climatic conditions. In Europe this might provide a sufficiently effective measure to reduce clinical disease to an acceptable level in small ruminants. Due to the fact that most cattle farmers in Europe are applying a year-round calving pattern in order to sustain a sufficient level of production and income a similar adjustment of insemination dates would however be highly economically disadvantageous.

Within this overall context, vaccines possess a number of convenient advantages: their effectiveness is independent of animal species or housing systems and vaccination requires only minor adaptions in herd or flock management. In addition, protection is reliable and long-standing (Kurogi, Inaba et al. 1978, Kim, Kweon et al. 2011). Mammalian cell lines are routinely employed for vaccine manufacturing (Barrett, Mundt et al. 2009). They allow the production of large amounts of viral antigen within a minimum of time. SBV has been demonstrated to replicate in a number of mammalian (and insect) cell-lines (Hoffmann, Scheuch et al. 2012, Elliott, Blakqori et al. 2013), including ones which have already been established for large scale vaccine production in different pharmaceutical companies (European Medicines Agency 2011). Based on these prerequisites, appropriate candidate cell-lines for the production of prototype SBV-vaccines could be chosen for the present studies.
Inactivation processes are another standard method in the production of so-called “killed vaccines” (Bahnemann 1990). These techniques can be adapted to a variety of viral pathogens as illustrated by the large range of commercially available inactivated vaccines. For the SBV-vaccines in our studies binary ethylenimine was applied as inactivating reagent.

In consequence, another important advantage of inactivated vaccines is that they can be produced relatively quickly and easily, which has also been demonstrated during the European BTV- and SBV-outbreaks.
Finally, inactivated vaccines are very safe as they do not contain infectious viruses and cannot replicate within the host, and thus do not cause disease and cannot infect arthropod vectors. They may also be applied in pregnant animals without hesitation. This has been demonstrated for example during safety tests with inactivated Akabane vaccines (Kurogi, Inaba et al. 1978, Kim, Kweon et al. 2011). Although this kind of safety data for inactivated SBV vaccines has so far only been made available for pregnant sheep (Moulin, Goovaerts et al. 2013), these preliminary data confirm that safety of inactivated SBV-vaccines is comparable to inactivated AKAV-vaccines. In the studies presented above it has furthermore been assumed that dissemination of the virus during viremia is necessary for efficient transplacental infection. As all but one of the tested prototype vaccines were able to prevent viremia completely – as measured by a very sensitive screening for viral RNA in serum - it is improbable that fetal infection would have occurred if the vaccines had been tested in pregnant animals. Of course, this kind of safety testing has to be completed before these vaccines can be marketed with the claim of fetal protection. All the advantages listed above make inactivated vaccines the first choice in case of an emergency like the recent SBV-epidemic
Unfortunately, our studies have shown that inactivated vaccines for the orthobunyaviruses AKAV and AINOV are not suitable for prevention of SBV infection due to insufficient antigenic relationship. They could have provided the possibility to initiate immediate counteractions to control the SBV-epidemic.
On the other hand, we have demonstrated that SBV-specific inactivated vaccines successfully prevent SBV-infection in susceptible hosts without causing adverse effects. Additional verification of these results is awaited from field application of those vaccines which have already been provisionally licensed in France (SBVvax, Mérial) and Great Britain (Bovlis SBV, MSD Animal Health).

In the present study, binary ethyleneimine (BEI) was chosen as inactivating agent instead of formalin, which was employed in inactivated vaccines for related orthobunyaviruses like AKAV (Kurogi, Inaba et al. 1978, Kim, Kweon et al. 2011). Formalin inactivates viral particles by cross-linking of proteins. This alters the conformational structure of surface glycoproteins (Brown 2001). Thus, the virus is not able to attach to its cellular receptors and infection of cells is prevented. One difficulty which may be associated with formalin is that the chemical reaction may also affect antigenic structures (Brown 2001) , i.e. structures which may be attacked by antibodies. If epitope conformation differs significantly between vaccine virus and wild-type virus, the effectiveness of the host's humoral immune response against wild-type epitopes may be impaired. In contrast, with BEI the inactivating activity of the agent is deemed to be associated mostly with structural changes in the viral nucleic acid (Brown 2001). Thus, antigenic structures are supposed to be better conserved. However, protein modifications by BEI cannot be excluded (Käsermann, Wyss et al. 2001).

A number of variations of the BEI-based inactivation process have been tested for SBV and the efficacy of the resulting vaccine preparations has been compared. Although our studies were primarily intended to serve as a proof of concept, our results indicate that factors like the production cell-line, duration of the inactivation reaction and the viral titer prior to inactivation do have an influence on vaccine performance. The mechanism by which the cell-line used for virus propagation might influence the performance of the resulting vaccine is not clear. On the other hand, it seems easily conceivable that the time of contact between virus and inactivating agent is crucial in this context. Based on the nature of the chemical reaction described above, inactivation of the virus might be incomplete if incubation is too short. In consequence, infectious virus would remain in the final product. Fortunately, the success of the inactivation reaction can easily be assessed, e.g. by passaging of a sample on a permissive cell-line. Appropriate tests are routinely performed during production of inactivated vaccines and are mandatory according to the European Directive 2001/82/EC, Annex I, Title II, Part 2, D.

Very long incubation times lead to accumulation of chemical modifications in viral molecules and an increased risk of impairment of antigenic structures as mentioned above. Such impairment is difficult to detect. Adaptive immunity is a complex system influenced by many factors which are not always easily identified. In general, animal trials are recommended for analysis and comparison of vaccine-induced immune responses. Especially challenge experiments, like the ones conducted in the present studies, are recommended to assess a vaccine's protective efficacy (OIE World Organisation for Animal Health 2014). In our case, the assessment of vaccine effectiveness was based on measurements of the humoral immune response (antibody titers) and on the extent to which vaccination was able to reduce viral replication in the host (presence of viral RNA in serum or organ material). In our case these criteria turned out to be adequate markers for vaccine performance as presence of antibodies correlated well with absence of viral replication after challenge infection. Furthermore, prevention of viremia must be considered an important aspect for a virus which is transmitted by haematophagous arthropods.

Finally, it seems logical that the amount of antigen, which is administered per dose of vaccine, contributes to vaccine efficacy. As the vaccine virus is inactivated and will not replicate within the host animal, the amount of antigen which is administered will not increase further but will only be degraded by cells of the innate immune system like macrophages, neutrophils or dendritic cells. Phagocytosis of the foreign material and subsequent presentation of these antigens to further immune cells (B- and T-lymphocytes) – especially in the presence of further immune-stimulating substances like adjuvants – on the other hand initiates an adaptive immune response (McHeyzer-Williams and McHeyzer-Williams 2005, Kaech and Wherry 2007). The amplitude of the final response, however, is dependent on the number of cells which were initially activated by contact to the specific antigen (Kedl, Rees et al. 2000, Wherry, McElhaugh et al. 2002).

Overall, we could observe that one of the vaccines which we tested, was not able to entirely prevent the development of RNAemia. However, it is difficult to differentiate clearly between inactivation effects, dose-dependent effects or cell-line effects and furthermore an influence

of the targeted animal species cannot be excluded. More detailed studies with a larger number of experimental animals would be necessary to elucidate this matter. However, this was not within the scope of our studies.
We can conclude as a rule of thumb that the antigen amount of the vaccine should not be too low, that inactivation time should be as short as possible for a given concentration of the inactivating agent and that the influence of the production-cell line should be analyzed prior to the start of large-scale production. Furthermore, vaccine-producers will want to determine the lowest amount of antigen per dose, which is able to confer reliable protection in order to obtain a maximum of doses from the given amount of virus that can be produced within a certain amount of time. In this context, it is of particular interest that even a single immunization is able to protect target animals from infection as it has been demonstrated here for sheep.

Currently, the level of herd immunity in the majority of the European livestock population is high. Consequently, the effort needed to sustain it will be relatively low. This would be beneficial in order to counteract potential future outbreaks. To this aim, SBV-specific vaccination would be of special value in heifers or young sheep or goats prior to the first mating. In this way detrimental effects of natural infection can be avoided (fertility problems, fetal death, losses of newborns or dams) and an important factor for reduction of herd-immunity (immunologically naïve, female offspring) is abolished. Such a risk-based immunization of selected animals is also cost-effective. However, against the background of a long-term decrease in antibody titers – both after natural infection and vaccination – sero-prevalences should best be monitored so that animals being at risk of reinfection may be recognized in time. Beyond that, a European-wide monitoring program for SBV (and potentially other arboviruses) following the example of the Australian NAMP (National Arbovirus Monitoring Program) (Animal Health Australia 2013) could prove useful for judging the overall need for control measures.
Secondly, trade restrictions constitute an important component of the economic impact of SBV-circulation in Europe (European Food Safety Authority 2014). Purchase of live animals or other biological material like embryos or semen by non-EU countries has declined considerably due to fear of SBV-introduction through these routes. In this context vaccination programs could be a provision to regain trust. To this aim, however, further data will have to be collected. At present, there is no indication that an animal, which has been infected by SBV and has detectable antibody titers, is at risk of being re-infected or presents a risk as source of further transmission. However, these assumptions have not been fully proven, yet. Especially, the duration of immunity after vaccination has not been studied in detail and thus the time period after which revaccination is needed can only be estimated. Furthermore, it is not clear if the virus which can be found in lymphatic and other tissues after infection could be a source for reactivation of infection in case of immunosuppression. Such a scenario would, of course, bear the risk that seemingly immune animals could become a source for virus transmission which entails unpredictable consequences for holdings buying such animals. Generally spoken, it has to be investigated if animals possessing anti-SBV antibodies present a risk for potential purchasers in order to enable trade also with vaccinated animals.

4.3 Options for further enhancement of SBV-vaccines

Thus far, it has been described that conventional inactivated vaccines are versatile tools for disease control as they can be produced quickly and are highly safe. Beyond that, the available vaccines based on field strains could be further enhanced by the use of DIVA-compatible (Differentiation of Infected from Vaccinated Animals) virus strains. Although appropriate viruses would have to be designed first, DIVA vaccination along with suitable diagnostic systems would offer additional security in international trade as animals can be proven free from natural infection.
On the other hand, inactivated vaccines are also associated with some disadvantages. Their capability to stimulate the immune system is essentially dependent on adjuvants (Cox and Coulter 1997). Additionally, inactivated vaccines often require the administration of more than one dose in order to confer protective immunity although the data presented in this work demonstrate that this is not entirely true for SBV-specific vaccines as a single immunization was able to prevent viremia and infection of lymphatic target organs in sheep. However, regular revaccination is necessary (Anonymous 1983). One reason for this is that the inactivation process involves a certain extent of protein modification, which may impair antigen quality and thus affects the affinity of pathogen-specific antibodies as has been described above (Brown 2001, Käsermann, Wyss et al. 2001). On the other hand stimulation of the host's immune system is reduced if the pathogen is not replicating. This can also be observed in our experimental studies. The immunogenicity of replicating live virus like the one used in control animals clearly exceeds that of the inactivated vaccines as measured by antibody response. I.e. live virus more quickly elicits higher antibody titers. Additionally, replicating virus will elicit not only a humoral but also a cellular immune response, including cytotoxic T-cells, which are an indispensable component of the host's natural defense against viral infections as they are capable of identifying and destroying infected cells thereby drastically reducing the production of new infectious particles (Rouse, Norley et al. 1988, Kaech, Wherry et al. 2002).
In summary, modified live vaccines could render vaccine application a lot more user friendly as the number of vaccinations could be reduced and vaccination intervals could be elongated while at the same time protection would be enhanced. Of course, appropriate virus strains have to be generated and characterized first. Furthermore, rigorous safety-tests must be conducted with relevant candidate strains. Especially the possibility of transplacental infection in pregnant vaccinees should be excluded. This has been a problem with modified live viruses used for immunization against RVFV or AKAV, for example (Hashiguchi, Murakami et al. 1981, Anonymous 1983). Additionally, the vaccine virus should be designed in a way which reduces the risk of reversion to virulence to a minimum. Otherwise, the vaccine virus itself could cause new outbreaks (Anonymous 1983).

Another important issue will be the long-term genetic stability of SBV and corresponding prospects for antigenic variations and vaccine efficacy. As detailed below, first insights in the genetic variability of SBV have been gathered. However, long-term prospects can only be deduced from the knowledge gathered about AKAV. In order to assess the extent of genetic

variation of AKAV various isolates obtained in Asia (Japan, Korea, Taiwan), Australia, Africa and Israel at different points of time were sequenced (Yamakawa, Yanase et al. 2006, Kobayashi, Yanase et al. 2007, An, Yoon et al. 2010). Additionally, antigenic relationships of the different viral strains have been analyzed (Akashi and Inaba 1997, Yoshida and Tsuda 1998).

In general AKAV strains can be assigned to 4 genogroups according to the nucleotide sequences of their M or S segments and the relationship of the different isolates is clearly influenced by time and location of isolation (Yamakawa, Yanase et al. 2006, Kobayashi, Yanase et al. 2007). Besides finding various mutations within the nucleotide sequences the studies also indicate the possibility of reassortment events.

It is hardly surprising that these genetic variations are mirrored on the protein level. Comparison of different isolates in respect to their reactivity with panels of monoclonal antibodies demonstrated changes in epitope structures (Akashi and Inaba 1997, Yoshida and Tsuda 1998) which in turn may lead to reduced reactivity with antisera raised against classical vaccine strains (Ogawa, Fukutomi et al. 2007). What is more, the evolutionary process gave rise to strains with new pathogenic properties (Ogawa, Fukutomi et al. 2007, Oem, Yoon et al. 2012).

However, these changes are estimated to have taken several decades (Kobayashi, Yanase et al. 2007) and reactivity of recent isolates towards antisera of vaccinated animals has not vanished completely. The greater risk seems to emanate from incursions of viruses from other geographic regions, which differ markedly in their genetic and antigenic traits.

Preliminary results for SBV indicate a genetic stability similar to AKAV. Although numerous mutations can be found by sequencing SBV RNA (Rosseel, Scheuch et al. 2012, Fischer, Hoffmann et al. 2013, Hulst, Kortekaas et al. 2013) many of these mutants seem to be replication deficient as isolation on cell culture cannot be achieved. Another study demonstrates that significant numbers of mutations are also acquired during cell-culture passage (Coupeau, Claine et al. 2013). This seems to represent an adaption to cell culture, as the passaged viruses are able to grow to higher titers and form larger plaques. On the other hand, another study found that a viral isolate, which was passaged 32 times on CPT-Tert cells, very well changed its pathogenic traits (Varela, Schnettler et al. 2013). After all, a hypervariable region in the Gc glycoprotein could be identified (Coupeau, Claine et al. 2013, Fischer, Hoffmann et al. 2013) and the hypothesis has been put forward that this region is potentially involved in adaption to immune pressure. In general however, marked differences in the pathogenesis of SBV have not been observed so far in the field, although variations can be found in published sequences of different isolates. It has to be considered though, that detailed studies on the comparative characterization of different SBV-isolates in vitro or in experimental animals have not been published, yet. Thus, subtle variations could have simply been overlooked. In any case, it is recommendable to keep track of the genetic variability of SBV in the field in order to adjust future vaccination strategies in time.

5 Conclusion and Outlook

Schmallenberg virus specific inactivated vaccines have been proven to be safe and are efficaciously able to protect cattle and sheep from experimental infection. Beside the inactivated vaccines presented in this work two others have been developed and provisionally licensed in France and Great Britain, respectively. It will be informative to see how they will influence SBV-epidemiology in the coming years. A concerted action in SBV-disease control on a European level however, will be impossible if vaccine licensing is not completed in order to enable their use in further countries.
Of course, it will depend on the position which both, affected farmers and policy makers, will take towards the issue, if vaccine production is profitable for pharmaceutical companies. Against the background of high sero-prevalences and low numbers of clinical cases for example in Germany at the moment (Friedrich-Loeffler-Institut 2014) it seems justifiable just to wait and see how the situation is going to develop. It is unlikely however that this approach will be sufficient in the long term. Given the fact that the effectiveness of alternative preventive measures like housing, insecticide treatment and adjustment of mating periods or insemination dates is not proven beyond reasonable doubt and their applicability varies greatly between holdings, the value of vaccination should not be underestimated.
In order to enable informed decisions for all parties involved, it is furthermore important that the knowledge about the distribution and biology of SBV is continuously updated and relevant information is made publicly available.

In general we should keep in mind that efficient communication and cooperation across institutions and countries is one of the most powerful weapons against modern epidemics. This has been proven all around the world during outbreaks of both human diseases like MERS-Coronavirus and animal diseases like Bluetongue virus. The emergence of SBV is only the most recent example, which has once more demonstrated that a remarkable amount of knowledge can be gathered within a minimum of time. With ever increasing globalized flow of commodity and movement of travelers it will furthermore not have been the last time that we have to deal with an emerging “exotic” disease. Thus, we should continue to try and understand the mechanisms behind these processes in order to be even better prepared next time.

6 Summary

In summer 2011 an unknown pathogen emerged in Europe. It was quickly identified as a member of the Simbu serogroup, genus *Orthobunyavirus* within the *Bunyaviridae* family of viruses and named Schmallenberg virus (SBV) according to the location of its discovery. Similar to other representatives of the Simbu serogroup SBV-infection was shown to be transmitted by haematophagous midges and to cause fetal malformation, abortion, reduced fertility and mild febrile disease in cattle, sheep and goats. Furthermore, antibodies against the new virus were found in a wide range of wild ruminants. Monitoring of clinical disease in livestock and serological screening showed how SBV spread throughout the European continent with incredible speed. Based on the vector-borne nature of the virus and experiences with related viruses in Asia and Australia it was clear that vaccination would be the only possibility to effectively counteract the disease. The development of inactivated vaccines was launched quickly in different countries. In parallel, numerous research projects were brought on the way to learn more about the molecular biology, pathogenesis and epidemiology of SBV.

The present work contains detailed descriptions of the proof of concept studies which were conducted with various inactivated vaccines for prevention of SBV-infection. First, an inactivated vaccine containing antigen of the Simbu serogroup viruses Akabane virus and Aino virus, and the reovirus Chuzan virus was tested for its potential to protect cattle from an SBV-infection. Unfortunately, the vaccine proved to be ineffective due to insufficient antigenic relationship between SBV and its relatives. Next, several newly developed inactivated SBV-specific vaccines were evaluated. The preparations differed in respect to cell-lines used for virus growth, viral titer prior to inactivation and the inactivation protocol. Most of the candidate vaccines inhibited propagation of challenge virus in the host completely; one of them was at least able to significantly reduce viral replication after challenge infection. This was assessed by detection of viral RNA in post-challenge serum and in lymphatic organs sampled during autopsy. One of the SBV-specific vaccine candidates has furthermore proven to be effective even after a single application in sheep. Interestingly, two SBV-specific vaccine preparations similar to the ones evaluated in our studies have been provisionally licensed in Great Britain and France in 2013. Inactivated vaccines can be produced relatively easily and quickly and possess a well-established safety profile. Therefore, they represent an excellent tool in “emergency” situations like, in this case, the emergence of SBV. However, they are associated with some disadvantages. Immunogenicity may be suboptimal and duration of post-vaccination immunity is limited. Booster doses and regular revaccinations are necessary. For this reason attenuated live vaccines e.g. NSs deletion mutants or vectored vaccines, will potentially provide an essential supplementation to the existing inactivated vaccines. Overall, persistent circulation of the virus in Europe along with recurring outbreaks must be suspected. Monitoring of SBV’s epidemiology should therefore be continued and appropriate arrangements should be made to control future outbreaks, even if they may not reach the same magnitude as the past one.

7 Zusammenfassung

Im Sommer 2011 trat erstmals ein bis dahin unbekannter Krankheitserreger in Europa auf. Er wurde bald darauf als Mitglied der Simbu Serogruppe des Genus *Orthobunyavirus* in der Familie der *Bunyaviridae* identifiziert und nach dem Ort seiner Entdeckung als Schmallenberg-Virus (SBV) bezeichnet. Ähnlich wie andere Vertreter der Simbu Serogruppe wurde für SBV gezeigt, dass die Infektion durch blutsaugende Stechmücken übertragen wird und bei Rindern, Schafen und Ziegen zu fötalen Missbildungen, Aborten, Fruchtbarkeitsstörungen und milden fieberhaften Erkrankungen führt. Des Weiteren wurden Antikörper gegen das neue Virus bei einer Vielzahl von Wildwiederkäuern gefunden. Die Überwachung von Erkrankungsfällen bei Nutzieren und serologische Screenings zeigten, wie SBV sich mit unglaublicher Geschwindigkeit auf dem Europäischen Kontinent ausbreitete. Davon ausgehend, dass SBV von Vektoren übertragen wird, und basierend auf Erfahrungen mit verwandten Viren in Australien und Asien, war schnell klar, dass eine Impfung die einzige Möglichkeit sein würde, effektiv gegen die Erkrankung vorzugehen. Schnell wurde daher die Entwicklung inaktivierter Impfstoffe in mehreren Ländern gestartet. Parallel dazu wurden zahlreiche Forschungsprojekte auf den Weg gebracht, um mehr über die Molekularbiologie, Pathogenese und Epidemiologie des SBV in Erfahrung zu bringen.

Die vorliegende Arbeit beinhaltet detaillierte Beschreibungen der Studien zum Nachweis der Wirksamkeit verschiedener inaktivierter Vakzinen zur Vorbeugung von SBV Infektionen.

Zunächst wurde ein in Japan kommerziell erhältlicher inaktivierter Impfstoff, der Antigen von den Simbuviren Akabane- und Aino-Virus und dem Reovirus Chuzan-Virus enthält, auf seine Fähigkeit hin getestet, Rinder vor einer SBV Infektion zu schützen. Der Impfstoff erwies sich jedoch aufgrund unzureichender Übereinstimmung der antigenen Strukturen zwischen SBV und seinen Verwandten als unwirksam gegen SBV. Als nächstes wurden mehrere neu entwickelte, SBV-spezifische inaktivierte Impfstoffe getestet. Die Präparate unterschieden sich jeweils im Bezug auf die Zelllinie, die für die Virusvermehrung genutzt wurde, den Virustiter vor Inaktivierung und das Inaktivierungsprotokoll. Die meisten Impfstoffkandidaten verhinderten die Vermehrung von virulentem Virus im Wirt vollständig und einer der Impfstoffkandidaten erreichte zumindest eine signifikante Reduzierung der Virämie nach der Belastungsinfektion. Dies wurde anhand der Detektion von viraler RNA im Serum nach Belastungsinfektion und in Proben von lymphatischen Organen beurteilt, die bei der Sektion entnommen wurden. Die Wirksamkeit eines der SBV-spezifischen Impfstoffkandidaten konnte des Weiteren auch nach einmaliger Applikation in Schafen bewiesen werden. Interessanterweise sind 2013 zwei Impfstoffpräparate in Frankreich bzw. Großbritannien zugelassen worden, die denen ähneln, welche in unseren Studien getestet wurden.

Inaktivierte Impfstoffe können relativ schnell und einfach hergestellt werden und weisen ein sehr gutes Sicherheitsprofil auf. Daher stellen sie eine exzellente Option für „Notfallsituationen“ dar, wie in diesem Fall das Auftreten von SBV. Jedoch wohnen ihnen einige Nachteile inne. Zum Beispiel kann die Immunogenität reduziert sein und die Dauer der Immunität nach der Impfung ist unter Umständen begrenzt. Impfungen zur Verstärkung der Immunantwort und regelmäßige Nachimpfungen sind daher notwendig. Aus diesem Grund werden attenuierte Impfstoffe, wie z.B. NSs-Deletionsmutanten oder Vektorimpfstoffe

zukünftig möglicherweise eine entscheidende Ergänzung der existierenden inaktivierten Vakzinen liefern. Insgesamt muss zudem eine anhaltende Viruszirkulation in Europa, verbunden mit regelmäßig wiederkehrenden Krankheitsausbrüchen, befürchtet werden. Die Überwachung der Epidemiologie des SBV sollte daher fortgeführt werden und es sollten angemessene Vorkehrungen getroffen werden, um zukünftige Ausbrüche kontrollieren zu können, auch wenn sie möglicherweise nicht das selbe Ausmaß erreichen werden, wie der hinter uns liegende SBV-Ausbruch.

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9 Abbreviations

aa	amino acids
AHS	Arthrogryposis hydranencephaly syndrome
AINOV	Aino virus
AKAV	Akabane virus
BEI	Binary Ethylenimine
BHK	Baby Hamster Kidney (cell line)
BUNV	Bunyamavera virus
C.	Culicoides
C6/36	insect cell line derived from Stegomyia albopicta larvae
CF	complement fixation
CNS	central nervous system
CPT-Tert	cell line derived from choroid plexus of sheep
DIVA	Differentiation of Infected from Vaccinated Animals
DNA	desoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
ER	endoplasmatic reticulum
EU	European Union
HI	Haemagglutination inhibition
HmLu	cell line derived from Hamster Lung
ICTV	the International Committee on Taxonomy of viruses
KC	insect cell line derived from Culicoides sonorensis larvae
LSDV	Lumpy Skin Disease virus
M	medium
MERS	Middle East Respiratory Syndrome
mRNA	messenger RNA
NCR	non-coding region
NDV	Newcastle Disease virus
NS	non-structural protein
NT	neutralization test
nt	nucleotides
OIE	Office International des Epizooties, World Organisation for Animal Health
ORF	open reading frame
PCR	polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT-PCR	reverse transcription PCR
RVFV	Rift Valley Fever virus
S	small
SBV	Schmallenberg virus
SN	serum neutralization
VLP	virus-like particle

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